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(54) Title: VIRAL VARIANTS WITH ALTERED SUSCEPTIBILITY TO NUCLEOSIDE ANALOGS AND USES THEREOF

(57) Abstract: The present invention relates generally to viral variants exhibiting reduced sensitivity to particular agents and/or reduced interactivity with immunological reagents. More particularly, the present invention is directed to hepatitis B virus (HBV) variants exhibiting complete or partial resistance to nucleoside analogs and/or reduced interactivity with antibodies to viral surface components including reduced sensitivity to these antibodies. The present invention further contemplates assays for detecting such viral variants, which assays are useful in monitoring anti-viral therapeutic regimens and in developing new or modified vaccines directed against viral agents and in particular HBV variants. The present invention also contemplates the use of the viral variants to screen for agents capable of inhibiting infection, replication and/or release of the virus.

WO 03/066841 A1

# VIRAL VARIANTS WITH ALTERED SUSCEPTIBILITY TO NUCLEOSIDE ANALOGS AND USES THEREOF

## BACKGROUND OF THE INVENTION

### 5 FIELD OF THE INVENTION

The present invention relates generally to viral variants exhibiting reduced sensitivity to particular agents and/or reduced interactivity with immunological reagents. More particularly, the present invention is directed to hepatitis B virus (HBV) variants exhibiting 10 complete or partial resistance to nucleoside analogs and/or reduced interactivity with antibodies to viral surface components including reduced sensitivity to these antibodies. The present invention further contemplates assays for detecting such viral variants, which assays are useful in monitoring anti-viral therapeutic regimens and in developing new or modified vaccines directed against viral agents and in particular HBV variants. The present 15 invention also contemplates the use of the viral variants to screen for agents capable of inhibiting infection, replication and/or release of the virus.

### DESCRIPTION OF THE PRIOR ART

20 Bibliographic details of the publications referred to by author in this specification are collected at the end of the description.

The reference to any prior art in this specification is not, and should not be taken as, an acknowledgment or any form of suggestion that that prior art forms part of the common 25 general knowledge in any country.

Specific mutations in an amino acid sequence are represented herein as 'Xaa<sub>1</sub>nXaa<sub>2</sub>' where Xaa<sub>1</sub> is the original amino acid residue before mutation, n is the residue number and Xaa<sub>2</sub> is the mutant amino acid. The abbreviation 'Xaa' may be the three letter or single letter 30 (i.e. 'X') code. The amino acid residues for Hepatitis B virus DNA polymerase are numbered with the residue methionine in the motif Tyr Met Asp Asp (YMDD) being

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residue number 204 (Stuyver *et al.*, *Hepatology* 33: 751-757, 2001). The amino acid residues for hepatitis B virus surface antigen are number according to Norder *et al.* (*J. Gen. Virol.* 74: 341-1348, 1993).

- 5 The term nucleoside analogs has been used in reference to both nucleotide and nucleoside analogs.

Hepatitis B virus (HBV) can cause debilitating disease conditions and can lead to acute liver failure. HBV is a DNA virus which replicates *via* an RNA intermediate and utilizes  
10 reverse transcription in its replication strategy (Summers and Mason, *Cell* 29: 403-415, 1982). The HBV genome is of a complex nature having a partially double-stranded DNA structure with overlapping open reading frames encoding surface, core, polymerase and X genes. The complex nature of the HBV genome is represented in Figure 1. The polymerase consists of four functional regions, the terminal protein (TP), spacer, reverse transcriptase  
15 (rt) and ribonuclease (RNAse).

The polymerase gene of HBV overlaps the envelope gene, mutations in the catalytic domain of the polymerase can affect the amino acid sequence of the envelope protein and *vice versa*. In particular, the genetic sequence for the neutralization domain of HBV known  
20 as the 'a' determinant, which is found within the HBsAg and located between amino acids 99 and 169, actually overlaps the major catalytic regions of the viral polymerase protein and in particular domains A and B.

The presence of an HBV DNA polymerase has led to the proposition that nucleoside  
25 analogs could act as effective anti-viral agents. Examples of nucleoside analogs currently being tested are penciclovir and its oral form (FAM) [Vere Hodge, *Antiviral Chem Chemother* 4: 67-84, 1993; Boyd *et al.*, *Antiviral Chem Chemother.* 32: 358-363, 1987; Kruger *et al.*, *Hepatology* 22: 219A, 1994; Main *et al.*, *J. Viral Hepatitis* 3: 211-215, 1996] Lamivudine[(-)- $\beta$ -2'-deoxy-3'-thiacytidine; (3TC or LMV) [Severini *et al.*,  
30 *Antimicrobial Agents Chemother* 39: 1430-1435, 1995; Dienstag *et al.*, *New England J Med* 333: 1657-1661, 1995]. New nucleoside analogs which have already progressed to

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clinical trials include the pyrimidines Emtricitabine, ((-)- $\beta$ -L-2'-3'-dideoxy-5-fluoro-3'-thiacydidine; FTC), the 5-fluoro derivative of 3TC, and Clevudine (1-(2-fluoro-5-methyl- $\beta$ -L-arabino-furanosyl) uracil; L-FMAU), a thymidine analog. Like 3TC, these are pyrimidine derivatives with an unnatural "L"- configuration. Several purine derivatives have also progressed to clinical trials; they include Entecavir (BMS-200,475; ETV), a carbocyclic deoxyguanosine analog, diaminopurine dioxolane (DAPD), an oral pro-drug for dioxolane guanine ((-)- $\beta$ -D-2-aminopurine dioxolane; DXG) and Adefovir dipivoxil, an oral prodrug for the acyclic deoxyadenosine monophosphate nucleoside analog Adefovir (9-[phosphonyl-methoxyethyl]-adenine; PMEA).

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Whilst these agents are highly effective in inhibiting HBV DNA synthesis, there is the potential for resistant mutants of HBV to emerge during long term antiviral chemotherapy. In patients on prolonged LMV therapy key resistance mutations are selected in the rt domain within the polymerase at rtM204I/V +/- rtL180M. The nomenclature used for the polymerase mutations is in accordance with that proposed by Stuyver *et al.*, 2001, *supra*. Only LMV has been approved for use against chronic HBV infection. Lamivudine is a particularly potent inhibitor of HBV replication and reduces HBV DNA titres in the sera of chronically infected patients after orthotopic liver transplantation (OLT) by inhibiting viral DNA synthesis. LMV monotherapy seems unlikely to be able to control HBV replication in the longer term. This is because emergence of LMV-resistant strains of HBV seems almost inevitable during monotherapy and single therapy is generally inadequate to result in viral clearance *per se*.

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ETV is also a potent inhibitor of HBV replication. ETV is an orally available cyclopentyl deoxyguanosine analog with activity against hepadnaviruses and herpesviruses. Preclinical studies indicate that ETV is a highly potent inhibitor of HBV in enzyme- and cell-based assays (Innaimo *et al.*, *Antimicrobiol Agent Chem* 44: 1441-1448, 1997; Siefer *et al.*, *Antimicrobiol Agent Chem* 28: 3200-3208, 1998; Yamanaka *et al.*, *Antimicrobiol Agent Chem* 43: 190-193, 1999). ETV has also demonstrated efficacy against WHV in chronically-infected woodchucks (Colonno *et al.*, *JID* 184: 1236-45 2001; Genovesi *et al.*, *Antimicrobiol Agent Chem* 42: 3209-3217, 1998). A four week dose-escalation trial

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indicated that ETV was well-tolerated and resulted in a 2.5 log<sub>10</sub> mean reduction in viremia at the highest dose tested (1 mg/daily). LMV resistance mutations were reported to confer cross-resistance to ETV *in vitro*, although entecavir was still capable of inhibiting viral replication at higher doses; these data are somewhat surprising considering that ETV is not  
5 an L-nucleoside. ETV has been used successfully to treat patients with the LMV resistant HBV mutations. No specific ETV resistant mutations had been described.

Nucleoside analog therapy may be administered as monotherapy or combination therapy where two or more nucleoside analogs may be administered. The nucleoside analogs may  
10 also be administered in combination with other antiviral agents such as interferon or hepatitis B immunoglobulin (HBIG).

There is a need to identify nucleoside- and/or antibody-resistant variants of HBV. The rapid identification can lead to altered therapeutic protocols being pursued.

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**SUMMARY OF THE INVENTION**

The abbreviations defined in Table 1 are used in the subject specification

5

**TABLE 1***Abbreviations*

ABBREVIATION	DESCRIPTION
3TC	(LMV); (-)- $\beta$ -2'-deoxy-3'-thiacytidine
ADV	adefovir
DAPD	diaminopurine dioxolane
DXG	dioxolane guanine
ETV	entecavir
FAM	famciclovir
FTC	emtricitabine
HBIG	hepatitis B immunoglobulin
HBsAg	hepatitis B surface antigen
HBV	hepatitis B virus
LMV	lamivudine
PMEA	adefovir
RNAse	ribonuclease
rt	reverse transcriptase
YMDD	Tyr Met Asp Asp-a motif in the polymerase protein; where the Met residue is designated residue number 204 of the reverse transcriptase

Throughout this specification, unless the context requires otherwise, the word "comprise",  
10 or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

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Nucleotide and amino acid sequences are referred to by a sequence identifier number (SEQ ID NO:). The SEQ ID NOs: correspond numerically to the sequence identifiers <400>1, <400>2, etc. A sequence listing is provided after the claims.

- 5 The positions of nucleotide and amino acid mutations identified using nomenclature from genotypes B, C or F where the methionine residue in the YMDD motif of the DNA polymerase was designated position 550 (see Australian Patent No. 734831). The nucleotide and amino acid positions given in the present specification are based on a new nomenclature where the methionine residue in YMDD is position 204 and is referred to as
- 10 rtM204 where rt is an abbreviation for "reverse transcriptase".

- In accordance with the present invention, HBV resistant variants were identified in a patient (patient A) with chronic hepatitis B treated with both LMV and ETV and a liver transplant patient (patient B) treated with ETV that had been previously treated with a
- 15 number of nucleoside agents including LMV. In combination therapy, accordance with the present invention, resistant variants of HBV were identified, following LMV and ETV treatment, with mutations in the HBV DNA polymerase gene which reduce the sensitivity of HBV to these nucleoside analogs. Corresponding mutations in the surface antigen also occur. The identification of these HBV variants is important for the development of assays
- 20 to monitor LMV and/or ETV resistance and/or resistance to other nucleoside analog therapeutic regimes and to screen for agents which are useful as alternative therapeutic agents. The mutations detected in the HBV isolated from patient A in key functional domains namely the rtI169T + rtV173L + rtL180M + rtM204V is demonstrated to have reduced sensitivity to ETV in functional assays.

- 25 The detection of such HBV variants is particularly important in the management of therapeutic protocols including the selection of appropriate agents for treating HBV infection. The method of this aspect of the present invention is predicated in part on monitoring the development in a subject of an increased HBV load in the presence of a
- 30 nucleoside analog. The clinician is then able to modify an existing treatment protocol or select an appropriate treatment protocol accordingly.

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One aspect of the present invention, therefore, is directed to an isolated HBV variant comprising a nucleotide mutation in a gene encoding a DNA polymerase resulting in at least one amino acid addition, substitution and/or deletion to the DNA polymerase and which exhibits decreased sensitivity to ETV and/or LMV and optionally other nucleoside analogs. Preferably, the DNA polymerase exhibits reduced sensitivity to ETV, or and ETV. The variant HBV comprises a mutation in an overlapping open reading frame in its genome in a region defined by one or more of domains F and A through E of HBV DNA polymerase.

The present invention further contemplates a method for determining the potential for an HBV to exhibit reduced sensitivity to ETV and/or LMV or optionally other nucleoside analogs by isolating DNA or corresponding mRNA from the HBV and screening for a mutation in the nucleotide sequence encoding HBV DNA polymerase resulting in at least one amino acid substitution, deletion and/or addition in any one or more of domains F and A through E or a region proximal thereto of the DNA polymerase and associated with resistance or decreased sensitivity to ETV and/or LMV. The presence of such a mutation is an indication of the likelihood of resistance to said entecavir and/or LMV. Preferably, the HBV variant exhibits reduced sensitivity to ETV, or both ETV and LMV.

The present invention also provides a composition comprising a variant HBV resistant to ETV and/or LMV and optionally other nucleoside analogs or an HBV surface antigen from the variant HBV or a recombinant or derivative form thereof or its chemical equivalent and one or more pharmaceutically acceptable carriers and/or diluents. Yet another aspect of the present invention provides a use of the aforementioned composition or a variant HBV comprising a nucleotide mutation in a gene encoding a DNA polymerase resulting in at least one amino acid addition, substitution and/or deletion to the DNA polymerase and a decreased sensitivity to ETV and/or LMV and optionally other nucleoside analogs in the manufacture of a medicament for the treatment and/or prophylaxis of hepatitis B virus infection.



The present invention also contemplates a method for determining whether an HBV strain exhibits reduced sensitivity to a nucleoside analog by isolating DNA or corresponding mRNA from the HBV and screening for a mutation in the nucleotide sequence encoding the DNA polymerase wherein the presence of the following mutations in the spacer region and the rt region: spacerL97I, spacerK115R, spacerH116L, spacerL128F, spacerS137G, spacerR139G, spacerF142S, rtY54H, rtL91I, rtA97V, rtY124H, rtH126R, rtS135Y, rtI169T, rtV173L, rtL180M, rtM204V, rtA21S, rtA38E, rtF122L, rtT128N, rtQ130P, rtT184G, rtS202I, rtH248N, rtY252L or combinations thereof or an equivalent one or more other mutation is indicative of a variant which exhibits a decreased sensitivity to ETV and/or LMV and optionally other nucleoside analogs.

The subject method may also be practiced by screening for a mutation in the nucleotide sequence encoding the DNA polymerase wherein the presence of the following mutations in the B or C domain of the rt region: rtI169T, rtV173L, rtL180M, rtT184G, rtS202I, rtM204V or combinations thereof or an equivalent one or more other mutations is indicative of a variant which exhibits a decreased sensitivity to ETV and/or LMV and optionally other nucleoside analogs.

It should be noted that mutants rtV173L, rtL180M and rtM204V correspond to mutants V519L, L526M, M550V and M550V, respectively in Australian Patent No. 734831 (using an earlier nomenclature system).

Still a further methodology comprises screening for a mutation in the nucleotide sequence encoding the envelope genes wherein the presence of the following mutations in the PreS1, PreS2 and S genes (changes in the overlapping reverse transcriptase region are indicated in parenthesis): PreS1N114D, PreS1 T115S, PreS2 F22L, PreS2 V39A, PreS2 P52L, sL89V, sT118A, sF161L (= rtI169T), sE164D (= rtV173L), sI195M (= rtM204V), sI208T, PreS1 E86Q, PreS1 N91K, PreS2 P41H, sQ30K, sP120T, sL176V, sV194F or combinations thereof or an equivalent one or more other mutation is indicative of a variant which exhibits a decreased sensitivity to ETV and/or LMV and optionally other nucleoside analogs.

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Preferably, the variants are in isolated form such that they have undergone at least one purification step away from naturally occurring body fluid. Alternatively, the variants may be maintained in isolated body fluid or may be in DNA form. The present invention also

5 contemplates infectious molecular clones comprising the genome or parts thereof from a variant HBV. The detection of HBV or its components in cells, cell lysates, cultured supernatant fluid and bodily fluid may be by any convenient means including any nucleic acid-based detection means, for example, by nucleic acid hybridization techniques or *via* one or more polymerase chain reactions (PCRs). The term "bodily fluid" includes any fluid

10 derived from the blood, lymph, tissue or organ systems including serum, whole blood, biopsy and biopsy fluid, organ explants and organ suspension such as liver suspensions. The invention further encompasses the use of different assay formats of the nucleic acid-based detection means, including restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), single-strand chain polymorphism

15 (SSCP), amplification and mismatch detection (AMD), interspersed repetitive sequence polymerase chain reaction (IRS-PCR), inverse polymerase chain reaction (iPCR) and reverse transcription polymerase chain reaction (RT-PCR), amongst others. Reverse hybridization is a technique which is particularly useful in identifying specific nucleotides or nucleotide sequences. Other forms of detection include Northern blots, Southern blots

20 PCR sequencing, antibody procedures such as ELISA, Western blot and immunohistochemistry. A particularly useful assay includes the reagents and components required for immobilized oligonucleotide- or oligopeptide-mediated detection systems.

Another aspect of the present invention is directed to a variant HBV comprising a surface

25 antigen having an amino acid sequence with a single or multiple amino acid substitution, addition and/or deletion or a truncation compared to a surface antigen from a reference or wild type HBV and wherein an antibody generated to the reference or wild type surface antigen exhibits an altered immunological profile relative to said HBV variant. One altered profile includes a reduced capacity for neutralizing the HBV. More particularly, the

30 surface antigen of the variant HBV exhibits an altered immunological profile compared to a pre-treatment HBV where the variant HBV is selected for by a nucleoside analog of the

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HBV DNA polymerase. The variant HBV of this aspect of the invention may also comprise a nucleotide sequence comprising a single or multiple nucleotide substitution, addition and/or deletion compared to a pre-treatment HBV.

- 5 The present invention extends to an isolated HBsAg or a recombinant form thereof or derivative or chemical equivalent thereof corresponding to the variant HBV. Generally, the HBsAg or its recombinant or derivative form or its chemical equivalent comprises an amino acid sequence with a single or multiple amino acid substitution, addition and/or deletion or a truncation compared to an HBsAg from a reference HBV and wherein an
- 10 antibody directed to a reference HBV exhibits an altered immunological profile to an HBV carrying said variant HBsAg. In one embodiment, the altered immunological profile comprises a reduction in the ability to neutralize the variant HBV.

- The present invention is predicated in part on the identification and isolation of variants of
- 15 HBV that have a plurality of mutations and exhibit two or more characteristics selected from decreased or reduced sensitivity to one or more nucleoside analogs, a reduced level and/or functional activity of hepatitis B e antigen, or a reduced, abrogated or otherwise impaired immunological interactivity, relative to wild-type HBV. Thus, the identification of HBV variants with these mutational patterns is important *inter alia* for the development
- 20 of assays to detect HBV variants and assays to screen for agents which are useful in treating and/or preventing infections by those variants and/or other HBV isolates and for the development of alternative therapeutic regimes for managing HBV infections.

- Accordingly, one aspect of the present invention is directed to an isolated HBV variant
- 25 comprising a plurality of nucleotide mutations that correlate with at least two characteristics selected from (a) resistance to one or more nucleoside analogs, (b) a reduced level and/or functional activity of hepatitis B e antigen, or (c) a reduced, abrogated or otherwise impaired immunological interactivity.

- 30 Another aspect of the present invention contemplates an isolated HBV variant comprising a plurality of nucleotide mutations that correlate with (a) resistance to one or more

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nucleoside analogs, (b) a reduced level and/or functional activity of hepatitis B e antigen, and (c) a reduced, abrogated or otherwise impaired immunological interactivity.

Yet another aspect of the present invention provides an isolated HBV variant comprising a plurality of nucleotide mutations selected from two or more of (a) a nucleotide mutation in a gene encoding a DNA polymerase resulting in at least one amino acid addition, substitution and/or deletion to said DNA polymerase wherein said variant exhibits decreased sensitivity to ETV and/or LMV and optionally other nucleoside analogs, (b) a nucleotide mutation in a gene encoding a hepatitis B e antigen or in a transcriptional control element of said gene wherein said mutation results in a reduced level and/or functional activity of said hepatitis B e antigen, or (c) a nucleotide mutation in a gene encoding a hepatitis B polypeptide resulting in at least one amino acid addition, substitution and/or deletion to said polypeptide which reduces, abrogates or otherwise impairs its immunological interactivity.

Another aspect of the present invention contemplates a method for detecting an agent which exhibits inhibitory activity to an HBV by generating a genetic construct comprising a replication competent-effective amount of the genome from the HBV contained in a plasmid vector and then transfecting said cells with said construct, contacting the cells before, during and/or after transfection, with the agent to be tested, culturing the cells for a time and under conditions sufficient for the HBV to replicate, express genetic sequences and/or assemble and/or release virus or virus-like particles if resistant to said agents; and the subjecting the cells, cell lysates or culture supernatant fluid to viral- or viral-component-detection means to determine whether or not the virus has replicated, expressed genetic material and/or assembled and/or been released in the presence of the agent. In a preferred embodiment, the plasmid vector in a baculovirus vector and the method comprises generating a genetic construct comprising a replication competent-effective amount of the genome from the HBV contained in or fused to an amount of a baculovirus genome effective to infect cells and then infecting said cells with said construct, contacting the cells, before, during and/or after infection, with the agent to be tested, culturing the cells for a time and under conditions sufficient for the HBV to replicate, express genetic

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- sequences and/or assemble and/or release virus or virus-like particles if resistant to said agent and then subjecting the cells, cell lysates or culture supernatant fluid to viral- or viral-component-detection means to determine whether or not the virus has replicated, expressed genetic material and/or assembled and/or been released in the presence of the agent.

- In an alternative embodiment, the method comprises generating a continuous cell line comprising an infectious copy of the genome of the HBV in a replication competent effective amount such that said infectious HBV genome is stably integrated into said continuous cell line such as but not limited to 2.2.15 or AD, contacting the cells with the agent to be tested, culturing the cells for a time and under conditions sufficient for the HBV to replicate, express genetic sequences and/or assemble and/or release virus or virus-like particles if resistant to the agent and then subjecting the cells, cell lysates or culture supernatant fluid to viral- or viral-component-detection means to determine whether or not the virus has replicated, expressed genetic material and/or assembled and/or been released in the presence of the agent.

- In an alternative embodiment, the present invention also contemplate a method for detecting an agent which exhibits inhibitory activity to an HBV polymerase in an *in vitro* polymerase assay. The HBV polymerase activity can be examined using established assays (Gaillard *et al.*, *Antimicrob Agents Chemother.* 46(4): 1005-1013, 2002; Xiong *et al.*, *Hepatology.* 28(6): 1669-73, 1998). The HBV polymerase may be a wild-type or reference HBV polymerase or mutant HBV polymerase.

- In connection with these methods, the plasmid vector may include genes encoding part or all of other viral vectors such as baculovirus vectors or adenovirus vectors (see Ren and Nassal, *J. Virol.* 75(3): 1104-1116, 2001).

- The identification of viral variants enables the production of vaccines comprising particular recombinant viral components such as polymerases or envelope genes PreS1, PreS2, S encoding for L, M, S proteins as well as therapeutic vaccines comprising

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defective HBV variants. Rational drug design may also be employed to identify or generate therapeutic molecules capable of interacting with a polymerase or or envelope genes PreS1, PreS2, S encoding for L, M, S proteins or other component of the HBV. Such drugs may also have diagnostic potential.

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A summary of sequence identifiers used throughout the subject specification is provided in Table 2.

**TABLE 2***Summary of sequence identifiers*

10

SEQUENCE ID NO:	DESCRIPTION
1	region F of HBV DNA polymerase (Formula I)
2	regions A to E of HBV DNA polymerase (Formula II)
3	primer (OS1)
4	primer (TTA3)
5	primer (JM)
6	primer (ITA4)
7	primer (OS2)
8	primer SEQ2
9	primer TTA2
10	forward primer PC1
11	reverse primer PC2
12	HBV-specific molecule beacon primer
13-18	TR1 (Figure 4)
19-24	Pol Trans of TR1 (Figure 5)
25-30	HBsAg Trans of TR1 (Figure 6)
31	Pre-ETV (Figure 8)
32	On-ETV (Figure 8)
33	Pre-ETV (Figure 9)

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SEQUENCE ID NO:	DESCRIPTION
34	On-ETV (Figure 9)
35	Pre-ETV (Figure 10)
36	Post-ETV (Figure 10)

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**BRIEF DESCRIPTION OF THE FIGURES**

Figure 1 is a diagrammatic representation showing the partially double stranded DNA HBV genome showing the overlapping open reading frames encoding surface (S), core  
5 (C), polymerase (P) and X gene.

Figure 2 is graphical representation of Patient A's clinical history including therapy regimen, HBV DNA viral load and alanine transaminase (ALT) levels.

10 Figure 3 is a diagrammatic representation of the chemical structure of entecavir.

Figure 4 is a representation showing comparison of the HBV nucleotide sequence encoding the catalytic region of the polymerase gene in sequential samples from Patient A during LMV monotherapy or LMV/entecavir combination therapy.

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Figure 5 is a representation showing comparison of the deduced amino acid sequence of the catalytic region of the polymerase gene in sequential samples from Patient A during LMV monotherapy or LMV/entecavir combination therapy.

20 Figure 6 is a representation showing comparison of the deduced amino acid sequence of the envelope gene in sequential samples from Patient A during LMV monotherapy or LMV/entecavir combination therapy.

Figure 7 is a diagrammatic representation of a computer system for determining the  
25 potency value ( $P_A$ ) of a variant HBV.

Figure 8 is a representation showing comparison of the HBV nucleotide sequence encoding the catalytic region of the polymerase gene in sequential samples from Patient B during LMV monotherapy (prior to ETV) and on ETV therapy.

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Figure 9 is a representation showing comparison of the deduced amino acid sequence of the catalytic region of the polymerase gene in sequential samples from Patient B during LMV monotherapy (prior to ETV) and on ETV therapy.

- 5 Figure 10 is a representation showing comparison of the deduced amino acid sequence of the envelope gene in sequential samples from Patient B during LMV monotherapy (prior to ETV) and on ETV therapy.

- Figure 11 is a graphical representation of HBV DNA replicative intermediates detected by  
10 quantitative PCR relative to the no drug control for both wild type virus and the HBV encoding the mutations at rtI169T+rtV173L+rtL180M+rtM204V.

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## DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is predicated in part on the identification and isolation of nucleoside analog resistant variants of HBV following treatment of patients with ETV or LMV or ETV and LMV and optionally other nucleoside analogs. In particular, ETV, or ETV and LMV treated patients gave rise to variants of HBV exhibiting decreased or reduced sensitivity to ETV and/or LMV. Reference herein to "decreased" or "reduced" in relation to sensitivity to ETV and/or LMV includes and encompasses a complete or substantial resistance to the nucleoside analog as well as partial resistance and includes a replication rate or replication efficiency (yield phenotype) which is more than a wild-type in the presence of a nucleoside analog. In one aspect, this is conveniently measured by an increase in viral load to a level similar or greater than pre-treatment levels.

Accordingly, one aspect of the present invention is directed to an isolated HBV variant wherein said variant comprises a nucleotide mutation in a gene encoding a DNA polymerase resulting in at least one amino acid addition, substitution and/or deletion to said DNA polymerase and wherein said variant exhibits decreased sensitivity to ETV and/or LMV and optionally other nucleoside analogs.

Preferably, the decreased sensitivity is in respect of ETV, or both ETV and LMV.

In addition to a mutation in the gene encoding DNA polymerase, due to the overlapping nature of the HBV genome (Figure 1), a corresponding mutation may also occur in the gene encoding the surface antigen (HBsAg) resulting in reduced interactivity of immunological reagents such as antibodies and immune cells to HBsAg. The reduction in the interactivity of immunological reagents to a viral surface component generally includes the absence of immunological memory to recognize or substantially recognize the viral surface component. The present invention extends, therefore, to an HBV variant exhibiting decreased sensitivity to ETV and/or LMV and reduced interactivity of an immunological reagent to HBsAg wherein the variant is selected for following ETV and/or LMV combination or sequential treatment. The term "sequential" in this respect means ETV

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followed by LMV or LMV followed by ETV or multiple sequential administrations of each of ETV and LMV or LMV and ETV.

- 5 A viral variant may, therefore, carry mutation only in the DNA polymerase or both in the DNA polymerase and the HBsAg. The term "mutation" is to be read in its broadest context and includes multiple mutations.

- The present invention extends to a mutation and any domain of the HBV DNA polymerase and in particular regions F and A through E provided said mutation leads to decreased  
10 sensitivity to LMV and/or ETV. Region F of the HBV DNA polymerase is defined by the amino acid sequence set forth in Formula I [SEQ ID NO:1] below :

#### FORMULA I

- 15 L, B<sub>1</sub>, B<sub>2</sub>, D, W, G, P, C, B<sub>3</sub>, B<sub>4</sub>, H, G, B<sub>5</sub>, H, B<sub>6</sub>, I, R, B<sub>7</sub>, P, R, T, P, B<sub>8</sub>, R, V, B<sub>9</sub>, G, G, V, F, L, V, D, K, N, P, H, N, T, B<sub>10</sub>, E, S, B<sub>11</sub>, L, B<sub>12</sub>, V, D, F, S, Q, F, S, R, G, B<sub>13</sub>, B<sub>14</sub>, B<sub>15</sub>, V, S, W, P, K, F, A, V, P, N, L, B<sub>16</sub>, S, L, T, N, L, L, S\*

wherein:

20

- B<sub>1</sub> is L, or R, or I  
B<sub>2</sub> is E, or D  
B<sub>3</sub> is T, or D, or A, or N, or Y  
B<sub>4</sub> is E, or D  
25 B<sub>5</sub> is E, or K, or Q  
B<sub>6</sub> is H, or R, or N,  
B<sub>7</sub> is I, or T  
B<sub>8</sub> is A, or S  
B<sub>9</sub> is T or R  
30 B<sub>10</sub> is A, or T, or S  
B<sub>11</sub> is R, or T

- 19 -

- B<sub>12</sub> is V, or G  
 B<sub>13</sub> is S, or I, or T, or N, or V  
 B<sub>14</sub> is T, or S, or H, or Y  
 B<sub>15</sub> is R, or H, or K, or Q  
 5 B<sub>16</sub> is Q, or P;

and wherein S\* is designated as amino acid 74.

- In this specification, reference is particularly made to the conserved regions of the DNA  
 10 polymerase as defined by domains A to E. Regions A to E are defined by the amino acid  
 sequence set forth in Formula II [SEQ ID NO:2] below (and in Australian Patent No.  
 734831):

## FORMULA II

- 15 SZ<sub>1</sub>LSWLSLDVSAAFYHZ<sub>2</sub>PLHPAAMPHLLZ<sub>3</sub>GSSGLZ<sub>4</sub>RYVA  
 RLSSZ<sub>5</sub>SZ<sub>6</sub>Z<sub>7</sub>XNZ<sub>8</sub>QZ<sub>9</sub>Z<sub>10</sub>XXXZ<sub>11</sub>LHZ<sub>12</sub>Z<sub>13</sub>CSRZ<sub>14</sub>LYVSLZ<sub>15</sub>LLY  
 Z<sub>16</sub>TZ<sub>17</sub>GZ<sub>18</sub>KLHLZ<sub>19</sub>Z<sub>20</sub>HPIZ<sub>21</sub>LGFRKZ<sub>22</sub>PMGZ<sub>23</sub>GLSPFLLAQF  
 TSAIZ<sub>24</sub>Z<sub>25</sub>Z<sub>26</sub>Z<sub>27</sub>Z<sub>28</sub>RAFZ<sub>29</sub>H CZ<sub>30</sub>Z<sub>31</sub>FZ<sub>32</sub>YM\*DDZ<sub>33</sub>VLGAZ<sub>34</sub>Z<sub>35</sub>Z<sub>36</sub>  
 20 Z<sub>37</sub>HZ<sub>38</sub>EZ<sub>39</sub>LZ<sub>40</sub>Z<sub>41</sub>Z<sub>42</sub>Z<sub>43</sub>Z<sub>44</sub>Z<sub>45</sub>Z<sub>46</sub>LLZ<sub>47</sub>Z<sub>48</sub>GIHLNPZ<sub>49</sub>KT KR WGY  
 SLNFMGYZ<sub>50</sub>IG

wherein:

- 25 X is any amino acid;  
 Z<sub>1</sub> is N or D;  
 Z<sub>2</sub> is I or P;  
 Z<sub>3</sub> is I or V;  
 Z<sub>4</sub> is S or D;  
 30 Z<sub>5</sub> is T or N;  
 Z<sub>6</sub> is R or N;

- 20 -

- $Z_7$  is N or I;  
 $Z_8$  is N or Y or H;  
 $Z_9$  is H or Y;  
 $Z_{10}$  is G or R;  
5  $Z_{11}$  is D or N;  
 $Z_{12}$  is D or N;  
 $Z_{13}$  is S or Y;  
 $Z_{14}$  is N or Q;  
 $Z_{15}$  is L or M;  
10  $Z_{16}$  is K or Q;  
 $Z_{17}$  is Y or F;  
 $Z_{18}$  is R or W;  
 $Z_{19}$  is Y or L;  
 $Z_{20}$  is S or A;  
15  $Z_{21}$  is I or V;  
 $Z_{22}$  is I or L;  
 $Z_{23}$  is V or G;  
 $Z_{24}$  is C or L;  
 $Z_{25}$  is A or S;  
20  $Z_{26}$  is V or M;  
 $Z_{27}$  is V or T;  
 $Z_{28}$  is R or C;  
 $Z_{29}$  is F or P;  
 $Z_{30}$  is L or V;  
25  $Z_{31}$  is A or V;  
 $Z_{32}$  is S or A;  
 $Z_{33}$  is V or L or M;  
 $Z_{34}$  is K or R;  
 $Z_{35}$  is S or T;  
30  $Z_{36}$  is V or G;  
 $Z_{37}$  is Q or E;

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- Z<sub>38</sub> is L or S or R;  
Z<sub>39</sub> is S or F;  
Z<sub>40</sub> is F or Y;  
Z<sub>41</sub> is T or A;  
5 Z<sub>42</sub> is A or S;  
Z<sub>43</sub> is V or I;  
Z<sub>44</sub> is T or C;  
Z<sub>45</sub> is N or S;  
Z<sub>46</sub> is F or V;  
10 Z<sub>47</sub> is S or D;  
Z<sub>48</sub> is L or V;  
Z<sub>49</sub> is N or Q;  
Z<sub>50</sub> is V or I; and  
M\* is amino acid 204;

15

and wherein the first S is designated as amino acid 75.

Preferably, the mutation results in an altered amino acid sequence in any one or more of domains F and A through E or regions proximal thereto of the HBV DNA polymerase.

20

Another aspect of the present invention provides an HBV variant comprising a mutation in an overlapping open reading frame in its genome wherein said mutation is in a region defined by one or more of domains F and A through E of HBV DNA polymerase and wherein said variant exhibits decreased sensitivity to ETV and/or LMV and optionally  
25 other nucleoside analogs.

30

In a related embodiment, there is provided an HBV variant comprising a mutation in the nucleotide sequence encoding a DNA polymerase resulting in an amino acid addition, substitution and/or deletion in said DNA polymerase in one or more amino acids as set forth in Formulae I [SEQ ID NO:1] and/or II [SEQ ID NO:2]:

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## FORMULA I

L, B<sub>1</sub>, B<sub>2</sub>, D, W, G, P, C, B<sub>3</sub>, B<sub>4</sub>, H, G, B<sub>5</sub>, H, B<sub>6</sub>, I, R, B<sub>7</sub>, P, R, T, P, B<sub>8</sub>, R, V, B<sub>9</sub>, G, G, V,  
 F, L, V, D, K, N, P, H, N, T, B<sub>10</sub>, E, S, B<sub>11</sub>, L, B<sub>12</sub>, V, D, F, S, Q, F, S, R, G, B<sub>13</sub>, B<sub>14</sub>, B  
 5 15, V, S, W, P, K, F, A, V, P, N, L, B<sub>16</sub>, S, L, T, N, L, L, S\*

wherein:

- B<sub>1</sub> is L, or R, or I
- 10 B<sub>2</sub> is E, or D
- B<sub>3</sub> is T, or D, or A, or N, or Y
- B<sub>4</sub> is E, or D
- B<sub>5</sub> is E, or K, or Q
- B<sub>6</sub> is H, or R, or N,
- 15 B<sub>7</sub> is I, or T
- B<sub>8</sub> is A, or S
- B<sub>9</sub> is T or R
- B<sub>10</sub> is A, or T, or S
- B<sub>11</sub> is R, or T
- 20 B<sub>12</sub> is V, or G
- B<sub>13</sub> is S, or I, or T, or N, or V
- B<sub>14</sub> is T, or S, or H, or Y
- B<sub>15</sub> is R, or H, or K, or Q
- B<sub>16</sub> is Q, or P;

25

and

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## FORMULA II

SZ<sub>1</sub>LSWLSLDVSAAFYHZ<sub>2</sub>PLHPAAMPHELLZ<sub>3</sub>GSSGLZ<sub>4</sub>RYVA  
 RLSSZ<sub>5</sub>SZ<sub>6</sub>Z<sub>7</sub>XNZ<sub>8</sub>QZ<sub>9</sub>Z<sub>10</sub>XXXZ<sub>11</sub>LHZ<sub>12</sub>Z<sub>13</sub>CSRZ<sub>14</sub>LYVSLZ<sub>15</sub>LLY  
 5 Z<sub>16</sub>TZ<sub>17</sub>GZ<sub>18</sub>KLHLZ<sub>19</sub>Z<sub>20</sub>HPIZ<sub>21</sub>LGFRKZ<sub>22</sub>PMGZ<sub>23</sub>GLSPFLLAQF  
 TSAIZ<sub>24</sub>Z<sub>25</sub>Z<sub>26</sub>Z<sub>27</sub>Z<sub>28</sub>RAFZ<sub>29</sub>H CZ<sub>30</sub>Z<sub>31</sub>FZ<sub>32</sub>YM'DDZ<sub>33</sub>VLGAZ<sub>34</sub>Z<sub>35</sub>Z<sub>36</sub>  
 Z<sub>37</sub>HZ<sub>38</sub>EZ<sub>39</sub>LZ<sub>40</sub>Z<sub>41</sub>Z<sub>42</sub>Z<sub>43</sub>Z<sub>44</sub>Z<sub>45</sub>Z<sub>46</sub>LLZ<sub>47</sub>Z<sub>48</sub>GIHLNPZ<sub>49</sub>KTKRWGY  
 SLNFMGYZ<sub>50</sub>IG

10 wherein:

- X is any amino acid;
- Z<sub>1</sub> is N or D;
- Z<sub>2</sub> is I or P;
- 15 Z<sub>3</sub> is I or V;
- Z<sub>4</sub> is S or D;
- Z<sub>5</sub> is T or N;
- Z<sub>6</sub> is R or N;
- Z<sub>7</sub> is N or I;
- 20 Z<sub>8</sub> is N or Y or H;
- Z<sub>9</sub> is H or Y;
- Z<sub>10</sub> is G or R;
- Z<sub>11</sub> is D or N;
- Z<sub>12</sub> is D or N;
- 25 Z<sub>13</sub> is S or Y;
- Z<sub>14</sub> is N or Q;
- Z<sub>15</sub> is L or M;
- Z<sub>16</sub> is K or Q;
- Z<sub>17</sub> is Y or F;
- 30 Z<sub>18</sub> is R or W;
- Z<sub>19</sub> is Y or L;



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- $Z_{20}$  is S or A;  
 $Z_{21}$  is I or V;  
 $Z_{22}$  is I or L;  
 $Z_{23}$  is V or G;  
5  $Z_{24}$  is C or L;  
 $Z_{25}$  is A or S;  
 $Z_{26}$  is V or M;  
 $Z_{27}$  is V or T;  
 $Z_{28}$  is R or C;  
10  $Z_{29}$  is F or P;  
 $Z_{30}$  is L or V;  
 $Z_{31}$  is A or V;  
 $Z_{32}$  is S or A;  
 $Z_{33}$  is V or L or M;  
15  $Z_{34}$  is K or R;  
 $Z_{35}$  is S or T;  
 $Z_{36}$  is V or G;  
 $Z_{37}$  is Q or E;  
 $Z_{38}$  is L or S or R;  
20  $Z_{39}$  is S or F;  
 $Z_{40}$  is F or Y;  
 $Z_{41}$  is T or A;  
 $Z_{42}$  is A or S;  
 $Z_{43}$  is V or I;  
25  $Z_{44}$  is T or C;  
 $Z_{45}$  is N or S;  
 $Z_{46}$  is F or V;  
 $Z_{47}$  is S or D;  
 $Z_{48}$  is L or V;  
30  $Z_{49}$  is N or Q;  
 $Z_{50}$  is V or I; and

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M\* is amino acid 204;

and wherein S\* in Formula I is designated as amino acid 74 and the first S in Formula II is designated as amino acid 75;

5

and wherein said variant exhibits decreased sensitivity to ETV and/or LTV and optionally other nucleoside analogs. Preferably, the decreased sensitivity is to ETV, or both LMV and/or ETV.

- 10 Another preferred aspect of the present invention contemplates an HBV variant comprising a mutation in the nucleotide sequence encoding HBsAg resulting in an amino acid addition, substitution and/or deletion in said HBsAg in a region corresponding to the amino acid sequence set forth in Formulae I and II wherein said variant exhibits decreased sensitivity to ETV and/or LMV and optionally other nucleoside analogs.

15

More particularly, the present invention provides a variant HBV comprising a surface antigen having an amino acid sequence with a single or multiple amino acid substitution, addition and/or deletion or a truncation compared to a surface antigen from a reference or wild type HBV and wherein an antibody generated to the reference or wild type surface

20 antigen exhibits reduced capacity for neutralizing said HBV variant, said variant selected by exposure of a subject to ETV and/or LMV in combination or sequential therapy.

- The term "combination therapy" means that both ETV and LMV are co-administered in the same composition or simultaneously in separate compositions. The term "sequential
- 25 therapy" means that the two agents are administered within seconds, minutes, hours, days or weeks of each other and in either order. Sequential therapy also encompasses completing a therapeutic course with one or other of ETV or LMV and then completing a second therapeutic course with the other of ETV or LMV.

- 30 Accordingly, another aspect of the present invention contemplates an HBV variant comprising a surface antigen having an amino acid sequence with a single or multiple

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amino acid substitution, addition and/or deletion or truncation compared to the pretreatment HBV and wherein the surface antigen of the variant HBV exhibits an altered immunological profile compared to the pretreatment HBV where the said the variant HBV is selected for by a nucleoside analog of the HBV DNA polymerase, said variant selected  
5 by exposure of a subject to ETV and/or LMV in combination or sequential therapy.

In a related embodiment, the present invention provides an HBV variant comprising a nucleotide sequence comprising a single or multiple nucleotide substitution, addition and/or deletion compared to the pretreatment HBV and which HBV variant has a surface  
10 antigen exhibiting an altered immunological profile compared to the pretreatment HBV, said variant selected by exposure of a subject to ETV and/or LMV in combination or sequential therapy.

Preferably, the variants are in isolated form such that they have undergone at least one  
15 purification step away from naturally occurring body fluid. Alternatively, the variants may be maintained in isolated body fluid or may be in DNA form. The present invention also contemplates infectious molecular clones comprising the genome or parts thereof from a variant HBV. Furthermore, the present invention provides isolated components from the variant HBVs such as but not limited to an isolated HBsAg. Accordingly, the present  
20 invention provides an isolated HBsAg or a recombinant form thereof or derivative or chemical equivalent thereof, said HBsAg being from a variant HBV selected by exposure of a subject to ETV and/or LMV in combination or sequential therapy.

More particularly, yet another aspect of the present invention is directed to an isolated  
25 variant HBsAg or a recombinant or derivative form thereof or a chemical equivalent thereof wherein said HBsAg or its recombinant or derivative form or its chemical equivalent exhibits an altered immunological profile compared to an HBsAg from a reference HBV, said HBsAg being from a variant HBV selected by exposure of a subject to ETV and/or LMV in combination or sequential therapy.

30

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Even more particularly, the present invention provides an isolated variant HBsAg or a recombinant or derivative form thereof or a chemical equivalent thereof wherein said HBsAg or its recombinant or derivative form or its chemical equivalent comprises an amino acid sequence with a single or multiple amino acid substitution, addition and/or deletion or a truncation compared to an HBsAg from a reference HBV and wherein a neutralising antibody directed to a reference HBV exhibits no or reduced neutralising activity to an HBV carrying said variant HBsAg, said HBsAg being from a variant HBV selected by exposure of a subject to ETV and/or LMV in combination or sequential therapy.

10

Preferred mutations in the HBV DNA polymerase include variants selected from patients with HBV recurrence following ETV and/or LMV treatment. Preferably, the treatment involves ETV or both ETV and/or LMV in combination or sequential therapy. Nucleoside analog treatment may occur in relation to a transplantation procedure (e.g. bone marrow transplantation (BMT) or OLT) or following treatment of patients diagnosed with hepatitis. Following selection of variants, viral loads are obtainable at levels greater than pre-treatment levels.

15

Preferred mutations in the HBV DNA polymerase include one or more of spacerL97I, spacerK115R, spacerH116L, spacerL128F, spacerS137G, spacerR139G, spacerF142S, rY54H, rL91I, rA97V, rY124H, rH126R, rS135Y, rI169T, rV173L, rL180M, rM204V, rA21S, rA38E, rF122L, rT128N, rQ130P, rT184G, rS202I, rH248N, rY252L or combinations thereof or an equivalent one or more other mutation is indicative of a variant wherein said variant exhibits a decreased sensitivity to ETV and/or LMV and optionally other nucleoside analogs. It should be noted that the nomenclature system for amino acid positions is based on the methionine residues in the YMDD motif being designated codon rM204. This numbering system is different to that in Australian Patent No. 734831 where the methionine residue in the YMDD motif within the polymerase gene is designated codon 550. In this regard, rV173L, rL180M and rM204V correspond to V519L, L526M and M550V, respectively, in Australian Patent No. 734831. The term "SPACER" means a region that has been designated between two functional regions:

25

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Terminal protein and reverse transcriptase. It provides the correct folding for the functional regions and no other specific function has been designated for this region. Corresponding mutations may also occur in envelope genes such as in one or more of PreS1, PreS2 and HBsAg. Particular mutations are as follows: PreS1 N114D, PreS1 T115S, PreS2 F22L, 5 PreS2 V39A, PreS2 P52L, sL89V, s T118A, s 161L, sE164D, sI195M, sI208T PreS1 E86Q, PreS1 N91K, PreS2 P41H, sQ30K, sP120T, sL176V, sV194F or combinations thereof or an equivalent one or more other mutation is indicative of a variant wherein said variant exhibits a decreased sensitivity to ETV and/or LMV and optionally other nucleoside analogs. The mutations in gene encoding HBsAg at sF161L, sE164D, or 10 sI195M also result in mutation in the in the polymerase gene rtI169T, rtV173L, or rtM204V respectively. Other corresponding mutations may occur in the rt such as spacerL97I, spacerK115R, spacerH116L, spacerL128F, spacerS137G, spacerR139G, spacerF142S, rtY54H, rtL91I, rtA97V, rtY124H, rtH126R, rtS135Y, rtI169T, rtV173L, rtL180M, rtM204V, rtA21S, rtA38E, rtF122L, rtT128N, rtQ130P, rtT184G, rtS202I, 15 rtH248N, rtY252L or combinations thereof or an equivalent one or more other mutation is indicative of a variant wherein said variant exhibits a decreased sensitivity to ETV and/or LMV and optionally other nucleoside analogs.

The identification of the variants of the present invention permits the generation of a range 20 of assays to detect such variants. The detection of such variants may be important in identifying resistant variants to determine the appropriate form of chemotherapy and/or to monitor vaccination protocols, or develop new or modified vaccine preparations.

Still another aspect of the present invention contemplates a method for determining the 25 potential for an HBV to exhibit reduced sensitivity to ETV and/or LMV or optionally other nucleoside analogs, said method comprising isolating DNA or corresponding mRNA from said HBV and screening for a mutation in the nucleotide sequence encoding HBV DNA polymerase resulting in at least one amino acid substitution, deletion and/or addition in any one or more of domains F and A through E or a region proximal thereto of said DNA 30 polymerase and associated with resistance or decreased sensitivity to ETV and/or LMV

- 29 -

wherein the presence of such a mutation is an indication of the likelihood of resistance to said ETV and/or LMV.

- Preferably, the assay detects one or more of the following mutations in the spacer region and/or the rt region: spacerL97I, spacerK115R, spacerH116L, spacerL128F, spacerS137G, spacerR139G, spacerF142S, rtY54H, rtL91I, rtA97V, rtY124H, rtH126R, rtS135Y, rtI169T, rtV173L, rtL180M, rtM204V, rtA21S, rtA38E, rtF122L, rtT128N, rtQ130P, rtT184G, rtS202I, rtH248N, rtY252L or combinations thereof or an equivalent one or more other mutation is indicative of a variant wherein said variant exhibits a decreased sensitivity to ETV and/or LMV and optionally other nucleoside analogs.

- Accordingly, another aspect of the present invention produces a method for determining whether an HBV strain exhibits reduced sensitivity to a nucleoside analog, said method comprising isolating DNA or corresponding mRNA from said HBV and screening for a mutation in the nucleotide sequence encoding the DNA polymerase wherein the presence of the following mutations in the spacer region and the rt region: spacerL97I, spacerK115R, spacerH116L, spacerL128F, spacerS137G, spacerR139G, spacerF142S, rtY54H, rtL91I, rtA97V, rtY124H, rtH126R, rtS135Y, rtI169T, rtV173L, rtL180M, rtM204V, rtA21S, rtA38E, rtF122L, rtT128N, rtQ130P, rtT184G, rtS202I, rtH248N, rtY252L or combinations thereof or an equivalent one or more other mutation is indicative of a variant wherein said variant exhibits a decreased sensitivity to ETV and/or LMV and optionally other nucleoside analogs.

- The preferred mutations in the reverse transcriptase are rtI169T, rtV173L, rtL180M, rtT184G, rtS202I, rtM204V or combinations thereof or an equivalent one or more other mutation is indicative of a variant wherein said variant exhibits a decreased sensitivity to ETV and/or LMV and optionally other nucleoside analogs.

- Accordingly, another aspect of the present invention contemplates a method for determining whether an HBV strain exhibits reduced sensitivity to a nucleoside analog, said method comprising isolating DNA or corresponding mRNA from said HBV and

- 30 -

- screening for a mutation in the nucleotide sequence encoding the DNA polymerase wherein the presence of the following mutations in the B or C domain of the rt region: rtI169T, rtV173L, rtL180M, rtT184G, rtS202I, rtM204V or combinations thereof or an equivalent one or more other mutation is indicative of a variant wherein said variant
- 5 exhibits a decreased sensitivity to ETV and/or LMV and optionally other nucleoside analogs.

- The detection of HBV or its components in cells, cell lysates, cultured supernatant fluid and bodily fluid may be by any convenient means including any nucleic acid-based
- 10 detection means, for example, by nucleic acid hybridization techniques or *via* one or more polymerase chain reactions (PCRs). The term "bodily fluid" includes any fluid derived from the blood, lymph, tissue or organ systems including serum, whole blood, biopsy and biopsy fluid, organ explants and organ suspension such as liver suspensions. The invention further encompasses the use of different assay formats of said nucleic acid-based detection
- 15 means, including restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), single-strand chain polymorphism (SSCP), amplification and mismatch detection (AMD), interspersed repetitive sequence polymerase chain reaction (IRS-PCR), inverse polymerase chain reaction (iPCR) and reverse transcription polymerase chain reaction (RT-PCR), amongst others. Other forms of detection include
- 20 Northern blots, Southern blots, PCR sequencing, antibody procedures such as ELISA, Western blot and immunohistochemistry. A particularly useful assay includes the reagents and components required for immobilized oligonucleotide- or oligopeptide-mediated detection systems.
- 25 One particularly useful nucleic acid detection system is the reverse hybridization technique. In this technique, DNA from an HBV sample is amplified using a biotin or other ligand-labeled primer to generate a labeled amplifcon. Oligonucleotides immobilized to a solid support such as a nitrocellulose film are then used to capture amplified DNA by hybridization. Specific nucleic acid fragments are identified *via* biotin
- 30 or the ligand. Generally, the labeled primer is specific for a particular nucleotide variation to be detected. Amplification occurs only if the variation to be detected is present. There

- 31 -

are many forms of the reverse hybridization assay and all are encompassed by the present invention.

Detecting HBV replication in cell culture is particularly useful.

5

Another aspect of the present invention contemplates a method for detecting an agent which exhibits inhibitory activity to an HBV by:

generating a genetic construct comprising a replication competent-effective  
10 amount of the genome from the HBV contained in a plasmid vector and then transfecting said cells with said construct;

contacting the cells, before, during and/or after transfection, with the agent to  
be tested;

15

culturing the cells for a time and under conditions sufficient for the HBV to replicate, express genetic sequences and/or assemble and/or release virus or virus-like particles if resistant to said agents; and

20

then subjecting the cells, cell lysates or culture supernatant fluid to viral- or viral-component-detection means to determine whether or not the virus has replicated, expressed genetic material and/or assembled and/or been released in the presence of the agent.

25

In a preferred embodiment, the plasmid vector may include genes encoding part or all of other viral vectors such as baculovirus or adenovirus (Ren and Nassal, 2001, *supra*) and the method comprises:

generating a genetic construct comprising a replication competent-effective  
30 amount of the genome from the HBV contained in or fused to an amount of a baculovirus



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genome or adenovirus genome effective to infect cells and then infecting said cells with said construct;

5       contacting the cells, before, during and/or after infection, with the agent to be tested;

          culturing the cells for a time and under conditions sufficient for the HBV to replicate, express genetic sequences and/or assemble and/or release virus or virus-like particles if resistant to said agent; and

10

          then subjecting the cells, cell lysates or culture supernatant fluid to viral- or viral-component-detection means to determine whether or not the virus has replicated, expressed genetic material and/or assembled and/or been released in the presence of the agent.

15

In an alternative embodiment, the method comprises:

          generating a continuous cell line comprising an infectious copy of the genome of the HBV in a replication competent effective amount such that said infectious HBV  
20       genome is stably integrated into said continuous cell line such as but not limited to 2.2.15 or AD;

          contacting the cells with the agent to be tested;

25

          culturing the cells for a time and under conditions sufficient for the HBV to replicate, express genetic sequences and/or assemble and/or release virus or virus-like particles if resistant to the agent; and

          then subjecting the cells, cell lysates or culture supernatant fluid to viral- or  
30       viral-component-detection means to determine whether or not the virus has replicated,

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expressed genetic material and/or assembled and/or been released in the presence of the agent.

As indicated above, variants may also be detected with reference to the HBsAg (s gene) and PreS1, PreS2 envelop genes. Preferred mutations in this regard include one or more of PreS1 N114D, PreS1 T115S, PreS2 F22L, PreS2 V39A, PreS2 P52L, sL89V, sT118A, sF161L, sE164D, sI195M, sI208T PreS1 E86Q, PreS1 N91K, PreS2 P41H, sQ30K, sP120T, sL176V, sV194F.

Accordingly, another aspect of the present invention contemplates a method for determining whether an HBV strain exhibits reduced sensitivity to a nucleoside analog, said method comprising isolating DNA or corresponding mRNA from said HBV and screening for a mutation in the nucleotide sequence encoding the envelope genes wherein the presence of the following mutations in the PreS1, PreS2 and HBsAg: PreS1 N114D, PreS1 T115S, PreS2 F22L, PreS2 V39A, PreS2 P52L, sL89V, sT118A, sF161L, sE164D, sI195M, sI208T PreS1 E86Q, PreS1 N91K, PreS2 P41H, sQ30K, sP120T, sL176V, sV194F or combinations thereof or an equivalent one or more other mutation is indicative of a variant wherein said variant exhibits a decreased sensitivity to ETV and/or LMV and optionally other nucleoside analogs.

The present invention is predicated in part on the identification and isolation of variants of HBV that have a plurality of mutations and exhibit two or more characteristics selected from decreased or reduced sensitivity to one or more nucleoside analogs, a reduced level and/or functional activity of hepatitis B e antigen, or a reduced, abrogated or otherwise impaired immunological interactivity, relative to wild-type HBV. Thus, the identification of HBV variants with these mutational patterns is important *inter alia* for the development of assays to detect HBV variants and assays to screen for agents which are useful in treating and/or preventing infections by those variants and/or other HBV isolates and for the development of alternative therapeutic regimes for managing HBV infections.

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Accordingly, one aspect of the present invention is directed to an isolated HBV variant comprising a plurality of nucleotide mutations that correlate with at least two characteristics selected from (a) resistance to one or more nucleoside analogs, (b) a reduced level and/or functional activity of hepatitis B e antigen, or (c) a reduced, abrogated or otherwise impaired immunological interactivity.

Another aspect of the present invention contemplates an isolated HBV variant comprising a plurality of nucleotide mutations that correlate with (a) resistance to one or more nucleoside analogs, (b) a reduced level and/or functional activity of hepatitis B e antigen, and (c) a reduced, abrogated or otherwise impaired immunological interactivity.

Yet another aspect of the present invention provides an isolated HBV variant comprising a plurality of nucleotide mutations selected from two or more of (a) a nucleotide mutation in a gene encoding a DNA polymerase resulting in at least one amino acid addition, substitution and/or deletion to said DNA polymerase wherein said variant exhibits decreased sensitivity to ETV and/or LMV and optionally other nucleoside analogs, (b) a nucleotide mutation in a gene encoding a hepatitis B e antigen or in a transcriptional control element of said gene wherein said mutation results in a reduced level and/or functional activity of said hepatitis B e antigen, or (c) a nucleotide mutation in a gene encoding a hepatitis B polypeptide resulting in at least one amino acid addition, substitution and/or deletion to said polypeptide which reduces, abrogates or otherwise impairs its immunological interactivity.

The detection of amino acid variants of DNA polymerase is conveniently accomplished by reference to the amino acid sequence shown in Formulae I and II. The polymorphisms shown represent the variations shown in various databases for active pathogenic HBV strains. Where an HBV variant comprises an amino acid different to what is represented, then such an isolate is considered a putative HBV variant having an altered DNA polymerase activity.

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The present invention further contemplates agents which inhibit ETV and/or LMV resistant HBV variants. Such agents will be particularly useful if long term treatment by ETV and/or LMV and/or optionally other nucleoside analogs is contemplated by the clinician. The agents may be DNA or RNA or proteinaceous or non-proteinaceous chemical molecules. Natural product screening such as from plants, coral and microorganisms is also contemplated as a useful potential source of masking agents. The agents may be in isolated form or in the form of a pharmaceutical composition and may be administered sequentially or simultaneously with the nucleoside analog.

10 Accordingly, another aspect of the present invention contemplates a method for detecting an agent which exhibits inhibitory activity to an HBV, exhibiting resistance or decreased sensitivity to ETV and/or LMV, said method comprising:

generating a genetic construct comprising a replication competent-effective  
15 amount of the genome from said HBV contained in a plasmid vector and then transfecting said cells with said construct;

contacting said cells, before, during and/or after transfection, with the agent  
to be tested;

20 culturing said cells for a time and under conditions sufficient for the HBV to replicate, express genetic sequences and/or assemble and/or release virus or virus-like particles if resistant to said agent; and

25 subjecting the cells, cell lysates or culture supernatant fluid to viral- or viral-component-detection means to determine whether or not the virus has replicated, expressed genetic material and/or assembled and/or been released in the presence of said agent.

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Still another aspect of the present invention provides a method for detecting an agent which exhibits inhibitory activity to an HBV, exhibiting resistance or decreased sensitivity to ETV and/or LMV, said method comprising:

5                   generating a genetic construct comprising a replication competent-effective amount of the genome from said HBV contained in or fused to an amount of a baculovirus genome effective to infect cells and then infecting said cells with said construct;

                  contacting said cells, before, during and/or after infection, with the agent to  
10   be tested;

                  culturing said cells for a time and under conditions sufficient for the HBV to replicate, express genetic sequences and/or assemble and/or release virus or virus-like particles if resistant to said agent; and

15                   subjecting the cells, cell lysates or culture supernatant fluid to viral- or viral-component-detection means to determine whether or not the virus has replicated, expressed genetic material and/or assembled and/or been released in the presence of said agent.

20   Still another aspect of the present invention provides a method for detecting an agent which exhibits inhibitory activity to an HBV, exhibiting resistance or decreased sensitivity to ETV and/or LMV, said method comprising:

                  generating a genetic construct comprising a replication competent-effective  
25   amount of the genome from said HBV contained in or fused to an amount of a baculovirus genome effective to infect cells and then infecting said cells with said construct;

                  contacting said cells, before, during and/or after infection, with the agent to  
                  be tested;  
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culturing said cells for a time and under conditions sufficient for the HBV to replicate, express genetic sequences and/or assemble and/or release virus or virus-like particles if resistant to said agent; and

- 5                   subjecting the cells, cell lysates or culture supernatant fluid to viral- or viral-component-detection means to determine whether or not the virus has replicated, expressed genetic material and/or assembled and/or been released in the presence of said agent.

Preferably, the HBV genome is stably integrated into the cells' genome.

10

Whilst the baculovirus vector is a particularly useful in the practice of the present invention, the subject invention extends to a range of other vectors such as but not limited to adenoviral vectors.

- 15   The present invention further extends to cell lines carrying genetic constructs comprising all or a portion of an HBV genome or a gene or part of a gene therefrom.

The present invention also provides for the use of the subject HBV variants to screen for anti-viral agents. These anti-viral agents inhibit the virus. The term "inhibit" includes  
20   antagonizing or otherwise preventing infection, replication, assembly and/or release or any intermediate step. Preferred anti-viral agents include nucleoside analogs, however, the present invention extends to non-nucleoside molecules.

- 25   In addition, rational drug design is also contemplated to identify or generate chemical molecules which either mimic a nucleoside or which interact with a particular nucleotide sequence or a particular nucleotide. Combinatorial chemistry and two hybrid screening are some of a number of techniques which can be employed to identify potential therapeutic or diagnostic agents.

- 30   In one example, the crystal structure of polymerase or the surface antigen is used to rationally design small chemical molecules likely to interact with key regions of the

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molecule required for function and/or antigenicity. Such agents may be useful as inhibitors of polymerase activity and/or may alter an epitope on the surface antigen.

- Several models of the HBV polymerase have been prepared due to the similarity with reverse transcriptase from HIV (Das *et al.*, *J. Virol.* 75(10): 4771-4779, 2001; Bartholomeusz *et al.*, *Intervirology* 40(5-6): 337-342 1997; Allen *et al.*, *Hepatology* 27(6): 1670-1677, 1998). The models of the HBV polymerase can be used for the rational drug design of new agents effective against HBV encoding the resistant mutations as well as wild type virus. The rational drug that is designed may be based on a modification of an existing antiviral agent such as the agent used in the selection of the HBV encoding the mutations associated with resistance. Viruses or clones expressing HBV genomic material encoding the mutations may also be used to screen for new antiviral agents.

- The above methods are particularly useful in identifying an inhibitor of a ETV- and/or LMV-resistant HBV. The present invention extends, therefore, to compositions of the inhibitors. The inhibitors may also be in the form of antibodies or genetic molecules such as ribozymes, antisense molecules and/or sense molecules for co-suppression or the induction of RNAi. Reference to RNAi includes reference to siRNA.

- The term "composition" includes a "pharmaceutical composition".

The inhibitor is referred to below as an "active ingredient" or "active compound" and may be selected from the list of inhibitors given above.

- The composition may include an antigenic component of the HBV, a defective HBV variant or an agent identified through natural product screening or rational drug design (including combinatorial chemistry).

- Pharmaceutically acceptable carriers and/or diluents include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active

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substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, use thereof in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

5

The pharmaceutical composition may also comprise genetic molecules such as a vector capable of transfecting target cells where the vector carries a nucleic acid molecule capable of encoding an aspartyl protease inhibitor. The vector may, for example, be a viral vector.

- 10 Pharmaceutical forms suitable for injectable use include sterile aqueous solutions (where water soluble) and sterile powders for the extemporaneous preparation of sterile injectable solutions. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dilution medium comprising, for example, water, ethanol,
- 15 polyol (for example, glycerol, propylene glycol and liquid polyethylene glycol, and the like), suitable mixtures thereof and vegetable oils. The proper fluidity can be maintained, for example, by the use of surfactants. The preventions of the action of microorganisms can be brought about by various anti-bacterial and anti-fungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal and the like. In many cases, it
- 20 will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminium monostearate and gelatin.
- 25 Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with the active ingredient and optionally other active ingredients as required, followed by filtered sterilization or other appropriate means of sterilization. In the case of sterile powders for the preparation of sterile injectable solutions, suitable methods of preparation include vacuum drying and the freeze-drying
- 30 technique which yield a powder of active ingredient plus any additionally desired ingredient.



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When the active ingredient is suitably protected, it may be orally administered, for example, with an inert diluent or with an assimilable edible carrier, or it may be enclosed in hard or soft shell gelatin capsule, or it may be compressed into tablets. For oral  
5 therapeutic administration, the active ingredient may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers and the like. Such compositions and preparations should contain at least 1% by weight of active compound. The percentage of the compositions and preparations may,  
10 of course, be varied and may conveniently be between about 5 to about 80% of the weight of the unit. The amount of active compound in such therapeutically useful compositions is such that a suitable dosage will be obtained. Preferred compositions or preparations according to the present invention are prepared so that an oral dosage unit form contains between about 0.1  $\mu\text{g}$  and 200 mg of active compound. Alternative dosage amounts  
15 dosages may be per individual or per kg body weight. Administration may be per hour, day, week, month or year.

The tablets, troches, pills, capsules and the like may also contain the components as listed hereafter. A binder such as gum, acacia, corn starch or gelatin; excipients such as  
20 dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; and a sweetening agent such as sucrose, lactose or saccharin may be added or a flavouring agent such as peppermint, oil of wintergreen or cherry flavouring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be  
25 present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills or capsules may be coated with shellac, sugar or both. A syrup or elixir may contain the active compound, sucrose as a sweetening agent, methyl and propylparabens as preservatives, a dye and a flavouring. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-  
30 toxic in the amounts employed. In addition, the active compound(s) may be incorporated into sustained-release preparations and formulations.

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As stated above, the present invention further extends to an isolated HBsAg from the HBV variants herein described. More particularly, the present invention provides an HBsAg or a recombinant form thereof or derivative or chemical equivalent thereof. The isolated surface component and, more particularly, isolated surface antigen or its recombinant, derivative or chemical equivalents are useful in the development of biological compositions such as vaccine formulations.

Yet another aspect of the present invention provides a composition comprising a variant HBV resistant to ETV and/or LMV and optionally other nucleoside analogs or an HBV surface antigen from said variant HBV or a recombinant or derivative form thereof or its chemical equivalent and one or more pharmaceutically acceptable carriers and/or diluents.

As indicated above, antibodies may be generated to the mutant HBV agents and used for passive or direct vaccination against infection by these viruses. The antibodies may be generated in humans or non-human animals. In the case of the latter, the non-human antibodies may need to be deimmunized or more specifically humanized prior to use. Deimmunized may include, for example, grafting complementarity determining regions (CDRs) from the variable region of a murine or non-human animal anti-HBV antibody onto a human consensus fragment antibody binding (Fab) polypeptide. Alternatively, amino acids defining epitopes in the variable region of the antibody may be mutated so that the epitopes are no longer recognized by the human MHC II complex.

Insofar as ribozyme, antisense or co-suppression (RNAi) repression is concerned, this is conveniently aimed at post-transcription gene silencing. DNA or RNA may be administered or a complex comprising RNAi or a chemical analog thereof specific for HBV mRNA may be employed.

All such molecules may be incorporated into pharmaceutical compositions.

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In another embodiment, the present invention provides a biological composition comprising a variant HBV or an HBsAg or L, M or S proteins from said variant HBV or a recombinant or derivative form thereof or its chemical equivalent.

- 5 Generally, if an HBV is used, it is first attenuated. The biological composition according to this aspect of the present invention generally further comprises one or more pharmaceutically acceptable carriers and/or diluents.

- 10 The biological composition may comprise HBsAg or like molecule from one HBV variant or the composition may be a cocktail of HBsAgs or L, M or S proteins or like molecules from a range of ETV- and/or LMV-resistant HBV variants. Similar inclusions apply where the composition comprises an HBV.

- 15 The present invention is further directed to the use of defective HBV variants in the manufacture of therapeutic vaccines to vaccinate individuals against infection by HBV strains having a particular nucleotide sequence or encoding a particular polymerase or surface antigen or L, M or S proteins.

- 20 In one embodiment, for example, an HBV variant may be identified having a particular mutation in its polymerase conferring resistance or decreased sensitivity to a nucleoside analog. This variant may then be mutated to render it defective, i.e. attenuated or unable to cause infection. Such a defective, nucleoside analog-resistant virus may then be used as a therapeutic vaccine against virulent viruses having the same mutation in its polymerase.

- 25 The subject invention extends to kits for assays for variant HBV resistant to ETV and/or LMV. Such kits may, for example, contain the reagents from PCR or other nucleic acid hybridization technology or reagents for immunologically based detection techniques. A particularly useful assay includes the reagents and components required for immobilized oligonucleotide- or oligopeptide-mediated detection systems.

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Still another aspect of the present invention contemplates a method for determining the potential for an HBV to exhibit reduced sensitivity to ETV and/or LMV or optionally other nucleoside analogs, said method comprising isolating DNA or corresponding mRNA from said HBV and screening for a mutation in the nucleotide sequence encoding HBV DNA polymerase resulting in at least one amino acid substitution, deletion and/or addition in any one or more of domains F and A through E or a region proximal thereto of said DNA polymerase and associated with resistance or decreased sensitivity to ETV and/or LMV wherein the presence of such a mutation is an indication of the likelihood of resistance to said ETV and/or LMV.

10

An assessment of a potential viral variant is important for selection of an appropriate therapeutic protocol. Such an assessment is suitably facilitated with the assistance of a computer programmed with software, which *inter alia* adds index values ( $I_{vs}$ ) for at least two features associated with the viral variants to provide a potency value ( $P_A$ ) corresponding to the resistance or sensitivity of a viral variant to a particular chemical compound or immunological agent. The  $I_{vs}$  can be selected from (a) the ability to exhibit resistance for reduced sensitivity to a particular compound or immunological agent; (b) an altered DNA polymerase from wild-type HBV; (c) an altered surface antigen from wild-type HBV; or (d) morbidity or recovery potential of a patient. Thus, in accordance with the present invention,  $I_{vs}$  for such features are stored in a machine-readable storage medium, which is capable of processing the data to provide a  $P_A$  for a particular viral variant or a biological specimen comprising same.

20

Thus, in another aspect, the invention contemplates a computer program product for assessing the likely usefulness of a viral variant or biological sample comprising same for determining an appropriate therapeutic protocol in a subject, said product comprising:

25

- (1) code that receives as input  $I_{vs}$  for at least two features associated with said viral agents or biological sample comprising same, wherein said features are selected from:

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- (a) the ability to exhibit resistance for reduced sensitivity to a particular compound or immunological agent;
- (b) an altered DNA polymerase from wild-type HBV;
- (c) an altered surface antigen from wild-type HBV; or
- 5 (d) morbidity or recovery potential of a patient;
- (e) altered replication capacity (increased or decreased);
- (2) code that adds said I<sub>vs</sub> to provide a sum corresponding to a P<sub>v</sub> for said viral variants or biological samples; and
- 10 (3) a computer readable medium that stores the codes.

In a related aspect, the invention extends to a computer for assessing the likely usefulness of a viral variant or biological sample comprising same in a subject, wherein said computer

15 comprises:

- (1) a machine-readable data storage medium comprising a data storage material encoded with machine-readable data, wherein said machine-readable data comprise I<sub>vs</sub> for at least two features associated with said viral variant or biological sample; wherein said features are selected from:-
- 20 (a) the ability to exhibit resistance for reduced sensitivity to a particular compound or immunological agent;
- (b) an altered DNA polymerase from wild-type HBV;
- 25 (c) an altered surface antigen from wild-type HBV; or
- (d) morbidity or recovery potential of a patient;
- (e) altered replication capacity (increased or decreased);
- (2) a working memory for storing instructions for processing said machine-readable data;
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- (3) a central-processing unit coupled to said working memory and to said machine-readable data storage medium, for processing said machine readable data to provide a sum of said  $I_V$ s corresponding to a  $P_V$  for said compound(s); and
- 5 (4) an output hardware coupled to said central processing unit, for receiving said  $P_V$ .

Any general or special purpose computer system is contemplated by the present invention and includes a processor in electrical communication with both a memory and at least one input/output device, such as a terminal. Such a system may include, but is not limited, to

10 personal computers, workstations or mainframes. The processor may be a general purpose processor or microprocessor or a specialized processor executing programs located in RAM memory. The programs may be placed in RAM from a storage device, such as a disk or pre-programmed ROM memory. The RAM memory in one embodiment is used both for data storage and program execution. The computer system also embraces systems where

15 the processor and memory reside in different physical entities but which are in electrical communication by means of a network. For example, a computer system having the overall characteristics set forth in Figure 7 may be useful in the practice of the instant invention. More specifically, Figure 7 is a schematic representation of a typical computer work station having in electrical communication (100) with one another *via*, for example, an

20 internal bus or external network, a processor (101), a RAM (102), a ROM (103), a terminal (104), and optionally an external storage device, for example, a diskette, CD ROM, or magnetic tape (105).

The present invention is further described by the following non-limiting Examples.

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**EXAMPLE 1*****Overlapping genome of HBV***

The overlapping genome of HBV is represented in Figure 1. The gene encoding DNA polymerase (P), overlaps the viral envelope genes, Pre-S1 and Pre-S2, and partially overlaps the X and core (C) genes. The HBV envelope comprises small, middle and large HBV surface antigens. The large protein component is referred to as the HBV surface antigen (HBsAg) and is enclosed by the S gene sequence. The Pre-S1 and Pre-S2 gene sequences encode the other envelope components.

10

**EXAMPLE 2*****Patient and Treatment***

Patient A is a 44 year old male with chronic hepatitis B presented on Day 0 (9 July 1999) with raised serum HBV DNA levels ( $>2000$  pg/ml) and was commenced on LMV treatment immediately (Figure 2). The patient A was HBsAg positive and anti-HBe positive. Following the initiation of LMV treatment the HBV DNA levels fell to 8 pg/ml over 54 days of therapy. The HBV DNA levels remained low until day 199 when there was a relapse in replication such that HBV DNA levels reached 1826 pg/ml. By Day 241 the serum ALT peaked at 741 IU/l. The HBV DNA was sequenced and LMV resistant virus was detected. The patient was then enrolled on Day 382 (24 July 2000) into a blinded ETV plus LMV clinical trial. HBV DNA levels only decreased to 33 pg/ml and the ALT decreased to 167 IU/L by day 784. The patient was started on open label ETV plus LMV. However, both HBV DNA levels and ALT continued to rise and the HBV DNA was sequenced at day 894 (Figure 2).

25

Patient B is a liver transplant patient. This patient has been treated with a number of nucleoside analogs including ganciclovir, famciclovir LMV and ETV. The patient was treated with LMV prior to ETV. The patient is currently on ETV treatment. During ETV treatment the HBV DNA levels were reduced to less than 5 pg/ml. At 532 days ETV treatment, that corresponds to 3857 days post transplant, there was a rise in the HBV DNA

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levels to 993 pg/ml. The HBV DNA from this sample was further characterized by sequencing.

### EXAMPLE 3

#### Detection of Viral Markers

- 5 Hepatitis B surface antigen (HBsAg), hepatitis B e antigen (HBeAg), anti-HBe and hepatitis B core antigen (HBcAg) specific IgG and IgM were measured using commercially available immunoassays (Abbott Laboratories, North Chicago, IL, USA).
- 10 Hepatitis B viral DNA levels were measured using a capture hybridization assay according to the manufacturer's directions (Digene Hybrid Capture II, Digene Diagnostics Inc., Beltsville, MD). The manufacturers stated cut-off for detecting HBV viremia in clinical specimens was  $0.7 \times 10^6$  copies/ml or 2.5 pg/ml, [Hendricks DA, *et al.*, *Am J Clin Pathol* 104: 537-46, 1995].

15

### EXAMPLE 4

#### Sequencing of HBV DNA

- HBV DNA was extracted from 100µl of serum collected at 6 different time points (Figure 2) as described previously by Aye *et al.*, *J Hepatol.* 26: 1148-53, 1997. Oligonucleotides were synthesized by Geneworks, Adelaide, Australia. Amplification of the HBV polymerase gene has been described by Aye *et al.*, 1997, *supra*.
- 20

- The specific amplified products were purified using PCR purification columns from MO BIO Laboratories Inc (La Jolla, CA) and directly sequenced using Big Dye terminator Cycle sequencing Ready Reaction Kit (Perkin Elmer, Cetus Norwalk, CT). The PCR primers were used as sequencing primers, OS1 5'- GCC TCA TTT TGT GGG TCA CCA TA -3' (nt 1408-1430) [SEQ ID NO: 3], TTA3 5'-AAA TTC GCA GTC CCC AAA - 3'(nt2128-2145) [SEQ ID NO: 4], JM 5'- TTG GGG TGG AGC CCT CAG GCT - 3'(nt1676-1696) [SEQ ID NO: 5], TTA4 5' -GAA AAT TGG TAA CAG CGG -3'(nt
- 25
- 30



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2615-2632) [SEQ ID NO: 6], OS2 5' TCT CTG ACA TAC TTT CCA AT 3' (nt 2798-2817) [SEQ ID NO: 7], to sequence the internal regions of the PCR products.

### EXAMPLE 5

#### *Analysis of HBV DNA*

5

Patient A: The LMV resistant mutations at rtL180M and rtM204V were detected by sequencing by day 199 (Table 3). During the blinded phase of entecavir and LMV treatment, the mutation at rtV173L was also detected. A unique mutation in the B Domain at rtI169T was detected in combination with the two other B domain mutations at rtL180M and rtV173L as well as the mutation at rtM204V in the C domain. A number of other unique changes were also detected in the polymerase and in the overlapping envelope gene (Table 4, Figures 4, 5 and 6). These unique changes were compared to reference sequences from each of the seven genotypes A-G as well as a consensus sequence from pretreatment samples to determine unique changes.

Patient B: The sample at 532 days ETV treatment was sequenced and was compared to samples prior to ETV treatment (Figures 8, 9 and 10) Several polymerase mutations were detected in this sample including rtA21S, rtA38E, rtY54H, rtN76D, rtL91I, rtF122L, rtY124H, rtT128N, rtQ130P, rtL180M, rtT184G, rtS202I, rtM204V, rtH248N, rtY252L. At the start of ETV treatment the patient had been on LMV treatment and the mutations at rtL80M and M204V were detected (Figures 8, 9 and 10). The LMV mutations (rtL180M and rtM204V) were detected during ETV treatment even in the absence of the LMV selection pressure and these mutations may also contribute to ETV resistance. At the time of the virological breakthrough on ETV, the LMV selected mutations were still present as well as the mutations listed above. All the mutations listed were compared to reference sequences from each of the seven genotypes A-G as well as a consensus sequence from pretreatment samples to determine unique changes.

Patient B is HBsAg negative and the HBV isolated from this patient encoded a mutation in the precore gene at G1896A that results in a stop codon in the precore protein

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precoreW28Stop. Mutations in other regions in the genome that included the precore mutation at G1896A may affect the replication fitness of HBV and the sensitivity to antiviral agents in combination with the mutation in the polymerase gene.

5

**EXAMPLE 6***In vitro analysis of entecavir resistance*

The sensitivity/resistance profile of HBV mutants to entecavir was examined *in vitro* using recombinant HBV/baculovirus. The procedure for analysing the resistance profile is  
10 outlined in the following Examples 7-14.

**EXAMPLE 7***Cell culture*

15 Sf21 insect cells were maintained in supplemented Grace's insect medium further supplemented with 10% v/v heat-inactivated fetal bovine serum (Gibco BRL, Gaithersburg, MD) in humidified incubator at 28 C with CO<sub>2</sub>. HepG2 cells were maintained in minimal essential medium supplemented with 10% v/v heat-inactivated fetal bovine serum (MEM-FBS). HepG2 cells were grown in humidified 37°C incubators at 5%  
20 v/v CO<sub>2</sub>.

**EXAMPLE 8***Preparation of HBV/baculovirus transfer vector with specific point mutations*

25 The recombinant HBV/baculovirus system used for antiviral testing has been previously described (Delaney *et al.*, *Antimicrob Agents Chemother* 45(6): 1705-1013, 2001). In brief, the recombinant transfer vector was created by excising a fragment containing the 1.3x HBV genome construct and cloning it into the multiple cloning region of a baculovirus vector pBlueBac4.5 (Invitrogen, Carlsbad, CA). Point mutations were created by site  
30 directed mutagenesis using the commercial kits according to the manufacturers specifications (QuikChange, Stratagene). A HBV recombinant encoding the reverse

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transcriptase mutations rtI169T + rtV173L + rtL180M + rtM204V. The nucleotide sequence of the plasmid and the point mutations generated by site directed mutagenesis were confirmed by sequencing using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit according to the manufacturer's specifications (Perkin

5 Elmer, Cetus Norwalk, CT).

### EXAMPLE 9

#### *Generation of recombinant baculoviruses containing the 1.3 HBV construct*

- 10 Purified recombinant transfer vector and linear AcMNPV baculovirus DNA were co-transfected into Sf21 cells using the BacNBlue transfection kit from Invitrogen (Carlsbad, CA); recombinant viruses were isolated by plaque assay according to the manufacturer's instructions. A series of recombinant viruses were amplified from isolated plaques by infecting 100-mm dishes of Sf21 cells. Viral DNA was extracted from amplified viruses
- 15 using standard procedures. Purified viral DNA was digested with restriction enzymes and then fractionated by electrophoresis in a 1% v/v agarose gel. Southern blotting was performed to determine which virus isolates contained the intact 1.3 HBV construct. A Boehringer Mannheim Random Prime DNA Labeling kit (Indianapolis, IN) was used to generate [ $P^{32}$ ]-radiolabeled probes. A full-length double-stranded HBV genome was used
- 20 as a template for all radiolabeled probes. Viral DNA sequence was confirmed by PCR amplification of the polymerase catalytic region using the sense primer 5'-GCC TCA TTT TGT GGG TCA CCA TA-3' [SEQ ID NO:3], (nucleotide 1408 to 1430 according to HBV Genebank Accession number M38454) and the antisense primer 5'-TCT CTG ACA TAC TTT CCA AT-3' [SEQ ID NO:6] (nucleotides 2817 to 2798 according to HBV Genebank
- 25 Accession number M38454). The following primers were utilized for the sequencing of internal regions 5'- TGC ACG ATT CCT GCT CAA-3' [SEQ ID NO:8] (nucleotides 2345-2362 according to HBV Genebank Accession number M38454) and 5'-TTT CTC AAA GGT GGA GAC AG-3' [SEQ ID NO:9] (nucleotides 1790-1810 according to HBV Genebank Accession number M38454).

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**EXAMPLE 10*****Preparative baculovirus amplification and purification***

Baculoviruses were amplified by infecting suspension cultures of Sf21 cells in log phase at a multiplicity of infection (moi) of 0.5 pfu/cell. Infections were allowed to proceed until a majority of the cells in the flasks showed visible signs of infection (four to five days). Virions were concentrated from infected Sf21 medium by centrifugation at 80,000 x g and purified through a 20-60% w/v sucrose gradient. Purified virus was titrated in quadruplicate in Sf21 cells by end-point dilution. An aliquot of each high titer stock was used for DNA extraction. The polymerase gene was amplified and sequenced to confirm the presence of the site-directed mutagenesis as in Example 9

**EXAMPLE 11*****Infection of HepG2 cells with recombinant HBV expressing baculovirus***

HepG2 cells were seeded at approximately 20-40% confluency and then were grown for 16-24 hours before infection. On the day of infection, triplicate plates of cells were trypsinized, and viable cell number was determined with a hemocytometer using Trypan blue exclusion. Average cell counts were calculated and used to determine the volume of high-titer viral stock necessary to infect cells at the indicated moi. HepG2 cells were washed one time with serum-free MEM to remove traces of serum. Baculovirus was diluted into MEM without serum to achieve the appropriate moi using volumes of 1.0, 0.5, and 0.25 ml to infect 100-mm, 60 mm, and 35-mm dishes, respectively. Baculovirus was adsorbed to HepG2 cells for one hour at 37°C with gentle rocking every 15 minutes to ensure that the inoculum was evenly distributed. The inoculum was then aspirated and HepG2 cells were washed two times with phosphate-buffered saline and refed MEM-FBS with or without various concentrations of agents.

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**EXAMPLE 12***Analysis of secreted HBV antigen*

Detection of hepatitis Be antigen (HBeAg) was performed by radioimmunoassay and  
5 microparticle enzyme immunoassay using kits purchased from Abbott Laboratories  
(Abbott Park, IL, USA). Medium from HepG2 cells was collected, centrifuged at 6,000 g  
to remove cellular debris, transferred to clean tubes, and stored at 20°C until analysis.  
HBeAg values are expressed as fold of positive control. Medium samples were diluted  
appropriately so that radioimmunoassay results were below positive control values for  
10 HBeAg.

**EXAMPLE 13***Detection of intracellular replicative intermediates*

15 HBV core particles were isolated from the cytoplasmic fraction of HepG2 cells lysed in  
0.5% w/v NP-40. Cytoplasmic extracts were adjusted to 10 mmol/l McC12 and  
unprotected DNA was removed by an incubation to 500 g/ml Proteinase K for 1.5 hours at  
37°C. HBV DNA in the samples were then extracted using commercial DNA extraction  
kits such as Qiagen (DNA extraction) or in-house methods using sequential phenol and  
20 chloroform extractions, and the nucleic acids were recovered by ethanol precipitation.  
Nucleic acids were resuspended in 50 µl /l TE (10 mmol/l Tris, 1 mmol/l  
ethylenediaminetetraacetic acid), normalized by OD260, and digested with 100 g/ml  
RNase (Boehringer Mannheim, Indianapolis, IN) for one hour at 37 °C before analysis by  
real-time PCR or electrophoresis and Southern blotting. After southern blot analysis a  
25 BioRad GS-670 imaging densitometer and the Molecular Analyst software (BioRad,  
Hercules California) was used to analyze suitable exposures of Southern blots.  
Densitometry data was fitted to logistic dose response curves using the TableCurve 2D  
software package from Jandel Scientific. Logistic dose response equations were used to  
calculate IC<sub>50</sub> and IC<sub>90</sub> values and co-efficients of variation.

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**EXAMPLE 14*****Real-time PCR***

- For the real-time PCR based assay for HBV, HBV DNA was extracted from 200 µl of serum using the QIAamp DNA Mini Kit according to the manufacturer's instructions (QIAGEN GmbH, Hildens, Germany). Primers and a molecular beacon were designed for conserved nucleic acid sequences within the precore domain of the HBV genome to amplify and detect a 216-nucleotide product (Figure 1). Amplification was performed in a 50-µl reaction mixture containing 1.0 Taqman buffer A (Applied Biosystems, Foster City, CA), 3.0 mM MgCl<sub>2</sub>, 0.4 pmol of each primer per µL, forward primer, PC1 (5'GGGAGGAGATTAGGTAA3' [SEQ ID NO:10]) and reverse primer, PC2 (5'GGCAAAAACGAGAGTAACTC3' [SEQ ID NO:11]), 0.4 pmol of the HBV-specific molecular beacon per µL, (5'FAM-CGCGTCCTACTGTTCAAGCCTCCAAGCTGTGACGCG-DABCYL-3' [SEQ ID NO:12]; where FAM represents fluorophore 6-carboxyfluorescein and DABCYL, 4-dimethylaminophenylazobenzoic acid, a quenching chromophore) and 1.25U of AmpliTaq Gold DNA polymerase (Perkin-Elmer). PCR was performed using the ABI PRISM 7700 spectrofluorometric thermocycler (Applied Biosystems). The PCR program consisted of an initial cycle (95°C for 10 minutes) followed by 45 amplification cycles (94°C for 15 secs, 50°C for 30 secs, 72°C for 30 secs). The instrument detected and recorded the fluorescence spectrum of each reaction tube during the annealing phase.

- An external standard was constructed by ligation of a 1.3 kB wild-type HBV plasmid (genotype D) into the pBlueBac plasmid vector (Hershey Medical Center, Hershey, PA). Quantification of the DNA concentration of the plasmid was determined by spectrophotometry. Duplicates of serial 10-fold dilutions of the plasmid ranging from 10<sub>3</sub> copies/ml to 100 copies/ml were included in each run in order to generate a standard curve. The copy number in each experimental reaction was determined by interpolation of the derived threshold cycle (C<sub>T</sub>).

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**EXAMPLE 15*****ETV treatments***

ETV was resuspended in sterile water, aliquoted, and frozen at -20°C to avoid repeated freezing and thawing of the drug. Medium containing ETV was prepared daily as needed using fresh aliquots of 3TC. In experiments in which ETV treatment was initiated after viral infection, HepG2 cells were exposed to the indicated concentration of ETV immediately after infection with HBV baculovirus. In experiments utilizing pretreatment with ETV, cells were fed medium containing ETV 16 hours prior to HBV baculovirus infection, HBV baculovirus infection was also carried out in medium containing ETV, and cells were refed fresh medium containing ETV immediately after completion of the infection and washing procedures.

**EXAMPLE 16**

***Antiviral testing performed with wild-type and HBV/baculovirus encoding  
rtIL69T + rtV173L + rtL180M + rtM204V***

The graphical analysis of the dose effect of ETV on wild-type HBV and the quadruple mutant HBV are shown in Figure 11 using the quantitative real-time PCR results relative to wild type virus grown in the absence of ETV (0  $\mu$ M ETV). ETV had the most pronounced effect on wild-type HBV replication as demonstrated by the reduction in HBV replicative intermediates detected by quantitative PCR at all ETV concentrations tested. In contrast, there was reduced sensitivity to ETV by the recombinant HBV encoding the quadruple mutant (rtI169T + rtV173L + rtL180M + rtM204V) especially at concentrations up to 0.5  $\mu$ M ETV.

**EXAMPLE 17*****ETV***

ETV (formerly BMS-200475 or SQ-34676) is a potent inhibitor of HBV replication. ETV is an cyclopentyl deoxyguanosine analog that has bio-oral available properties with activity

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against hepadnaviruses and herpesviruses. The structure of ETV is shown in Figure 3 and its synthesis is described by Bisacchi *et al.* (*Bioorg. Med. Chem. Lit.* 7: 127-132, 1997). Preclinical studies indicate that entecavir is a highly potent inhibitor of HBV in enzyme- and cell-based assays (Innaimo *et al.*, 1997, *supra*; Siefer *et al.*, 1998, *supra*; Yamanaka *et al.*, 1999, *supra*. ETV was formerly described as BMS-200475 and SQ-34676.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.



**TABLE 3** Clinical, virological and HBV sequencing data summary for the patient A with increasing HBV viral loads while on LMV and ETV

Days Post-LMV treatment	HBV DNA pg/ml	HBsAg	HBsAb	HBcAg	Anti-HBe	ALT IU/L	Treatment Protocol	Key Polymerase mutations detected by Sequencing
-143.00	>2000	detected				n/a		
-107.00				ND		399		
0.00							LMV (9/7/1999)	
54.00	8					510		
87.00	ND <sup>1</sup>					211		
115.00	ND			ND	+	106		
173.00	837					186		
199.00	1826					233		Sequenced: rL180M, rM204M/V <sup>2</sup>
241.00	930			ND	+	741		Sequenced: rL180M, rM204V
283.00	1631					334		
312.00	1539	+	ND	ND	+	238		
348.00	1605	+	ND	ND	+	349		
382.00	1303	+	ND	ND	+	317	ETV vs LMV <sup>2</sup> 24/7/2000	Sequenced rL180M, rM204V 00
397.00	131			ND	+	356		Sequenced rL180M, rM204V
495.00	44					205		
523.00	41					168		Sequenced rV173V/L rL180M, rM204V.
784.00	33	+	ND	ND	+	167	ETV plus LMV <sup>3</sup> 22/8/01	
798.00	117					190		
826.00	75	+	ND	ND	+	215		
882.00	362	+	ND	ND	+	392		
894.00	192					597		Sequenced rL169T, rV173L, rL180M rM204V
902.00	246					627		

1. ND= not detected

2. Blinded phase of the study

3. Open label phase of the study

4. Nomenclature according to Stuyver *et al.*, 2001, *supra*

TABLE 4 Summary of HBV mutations in patient A treated with ETV and LMV

Sample name	Sample date	Days post LMV treatment	PCR Status	Genotype	Polymerase*	Surface
TR1	24/01/2000	199	IR PCR +ve	D	rL180M** rM204V/M	sA/V177A/V sI195M/I
TR2	6/3/2000	241	IR PCR +ve	D	rL180M rV/M204V	sA/V177A/V sL193S sI/M195M
TR3	21/6/2000	348	2R PCR +ve	D	rL180M rM204V	sV/A177V sS193S/L sI/I195M
TR4	9/8/2000	397	2R PCR +ve	D	rL180M rM204V	sS/L177V sI195M
TR5	13/12/2000	523	2R PCR +ve	D	rV173V/L rL180M rM204V	sE164E/D sI195M
TR6	18/12/2001	894	IR PCR +ve	D	spacerL97I spacerK115R spacerH116L spacerL128F spacerS137G spacerR139G spacerF142S rY54H rY91I rA97V rY124H rH126R rS135Y rL69T rV173V/L rL180M rM204V	preS1 N114 preS1 T115S preS2 F22L preS2 V39A preS2 P52L sL89V sT118A sP127T sF161L sE164E/D sI/M195M

\* Nomenclature according to Stuyver *et al.*, 2001, *supra*

\*\* Mutations in bold have not been detected in reference HBV genotypes, mutations not in bold are changes from the previous sample that are present in reference genotypes

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**CLAIMS**

1. An isolated HBV variant wherein said variant comprises a nucleoside mutation in a gene encoding a DNA polymerase resulting in at least one amino acid addition, substitution and/or deletion to said DNA polymerase and wherein said variant exhibits decreased sensitivity to ETV and/or LMV and optionally other nucleoside analogs.
2. The isolated HBV variant of Claim 1 wherein said variant exhibits decreased sensitivity to ETV.
3. The isolated HBV variant of Claim 1 wherein said variant exhibits decreased sensitivity to both ETV and LMV.
4. The isolated HBV variant of any one of Claims 1 to 3 wherein said variant exhibits reduced interactivity to an immunological reagent specific to HBsAg.
5. The isolated HBV variant of Claim 1 wherein said variant comprises a mutation in domain F of the HBV DNA polymerase thereby conferring an altered amino acid sequence to the sequence set forth in Formula I (SEQ ID NO:1):

**FORMULA I**

L, B<sub>1</sub>, B<sub>2</sub>, D, W, G, P, C, B<sub>3</sub>, B<sub>4</sub>, H, G, B<sub>5</sub>, H, B<sub>6</sub>, I, R, B<sub>7</sub>, P, R, T, P, B<sub>8</sub>, R, V, B<sub>9</sub>, G, G, V, F, L, V, D, K, N, P, H, N, T, B<sub>10</sub>, E, S, B<sub>11</sub>, L, B<sub>12</sub>, V, D, F, S, Q, F, S, R, G, B<sub>13</sub>, B<sub>14</sub>, B<sub>15</sub>, V, S, W, P, K, F, A, V, P, N, L, B<sub>16</sub>, S, L, T, N, L, L, S\*

wherein:

B<sub>1</sub> is L or R or I;

B<sub>2</sub> is E or D;

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- B<sub>3</sub> is T or D or A or N or Y;  
 B<sub>4</sub> is E or D;  
 B<sub>5</sub> is E or K or Q;  
 B<sub>6</sub> is H or R or N;  
 B<sub>7</sub> is I or T;  
 B<sub>8</sub> is A or S;  
 B<sub>9</sub> is T or R;  
 B<sub>10</sub> is A or T or S;  
 B<sub>11</sub> is R or T;  
 B<sub>12</sub> is V or G;  
 B<sub>13</sub> is S or I or T or N or V;  
 B<sub>14</sub> is T or S or H or Y;  
 B<sub>15</sub> is R or H or K or Q;  
 B<sub>16</sub> is Q or P;

and wherein S\* is designated as amino acid 74.

6. The isolated HBV variant of Claim 1 wherein said variant comprises a mutation in any one of domains A through E thereby conferring an altered amino acid sequence to the sequence set forth in Formula II (SEQ ID NO:2):

## FORMULA II

S Z<sub>1</sub> L S W L S L D V S A A F Y H Z<sub>2</sub> P L H P A A M P H L L Z<sub>3</sub> G S S G L Z<sub>4</sub> R Y V A  
 R L S S Z<sub>5</sub> S Z<sub>6</sub> Z<sub>7</sub> X N Z<sub>8</sub> Q Z<sub>9</sub> Z<sub>10</sub> X X X Z<sub>11</sub> L H Z<sub>12</sub> Z<sub>13</sub> C S R Z<sub>14</sub> L Y V S L Z<sub>15</sub> L L Y  
 Z<sub>16</sub> T Z<sub>17</sub> G Z<sub>18</sub> K L H L Z<sub>19</sub> Z<sub>20</sub> H P I Z<sub>21</sub> L G F R K Z<sub>22</sub> P M G Z<sub>23</sub> G L S P F L L A Q F T  
 S A I Z<sub>24</sub> Z<sub>25</sub> Z<sub>26</sub> Z<sub>27</sub> Z<sub>28</sub> R A F Z<sub>29</sub> H C Z<sub>30</sub> Z<sub>31</sub> F Z<sub>32</sub> Y M\* D D Z<sub>33</sub> V L G A Z<sub>34</sub> Z<sub>35</sub> Z<sub>36</sub> Z<sub>37</sub>  
 H Z<sub>38</sub> E Z<sub>39</sub> L Z<sub>40</sub> Z<sub>41</sub> Z<sub>42</sub> Z<sub>43</sub> Z<sub>44</sub> Z<sub>45</sub> Z<sub>46</sub> L L Z<sub>47</sub> Z<sub>48</sub> G I H L N P Z<sub>49</sub> K T K R W G Y S L N  
 F M G Y Z<sub>50</sub> I G

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wherein:

X is any amino acid;  
Z<sub>1</sub> is N or D;  
Z<sub>2</sub> is I or P;  
Z<sub>3</sub> is I or V;  
Z<sub>4</sub> is S or D;  
Z<sub>5</sub> is T or N;  
Z<sub>6</sub> is R or N;  
Z<sub>7</sub> is N or I;  
Z<sub>8</sub> is N or Y or H;  
Z<sub>9</sub> is H or Y;  
Z<sub>10</sub> is G or R;  
Z<sub>11</sub> is D or N;  
Z<sub>12</sub> is D or N;  
Z<sub>13</sub> is S or Y;  
Z<sub>14</sub> is N or Q;  
Z<sub>15</sub> is L or M;  
Z<sub>16</sub> is K or Q;  
Z<sub>17</sub> is Y or F;  
Z<sub>18</sub> is R or W;  
Z<sub>19</sub> is Y or L;  
Z<sub>20</sub> is S or A;  
Z<sub>21</sub> is I or V;  
Z<sub>22</sub> is I or L;  
Z<sub>23</sub> is V or G;  
Z<sub>24</sub> is C or L;  
Z<sub>25</sub> is A or S;  
Z<sub>26</sub> is V or M;  
Z<sub>27</sub> is V or T;

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Z<sub>28</sub> is R or C;  
Z<sub>29</sub> is F or P;  
Z<sub>30</sub> is L or V;  
Z<sub>31</sub> is A or V;  
Z<sub>32</sub> is S or A;  
Z<sub>33</sub> is V or L or M;  
Z<sub>34</sub> is K or R;  
Z<sub>35</sub> is S or T;  
Z<sub>36</sub> is V or G;  
Z<sub>37</sub> is Q or E;  
Z<sub>38</sub> is L or S or R;  
Z<sub>39</sub> is S or F;  
Z<sub>40</sub> is F or Y;  
Z<sub>41</sub> is T or A;  
Z<sub>42</sub> is A or S;  
Z<sub>43</sub> is V or I;  
Z<sub>44</sub> is T or C;  
Z<sub>45</sub> is N or S;  
Z<sub>46</sub> is F or V;  
Z<sub>47</sub> is S or D;  
Z<sub>48</sub> is L or V;  
Z<sub>49</sub> is N or Q;  
Z<sub>50</sub> is V or I; and  
M\* is amino acid 204;

and wherein the first S is designated as amino acid 75.

7. The isolated HBV variant of Claim 5 or 6 wherein said variant further comprises an altered HBsAg.

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8. The isolated HBV variant of Claim 5 or 6 or 7 wherein said variant exhibits reduced interactivity to an immunological reagent specific to HBsAg.

9. An isolated HBV variant comprising a mutation in the nucleotide sequence encoding HBsAg resulting in an amino acid addition, substitution and/or deletion in said HBsAg in a region corresponding to the amino acid sequence set forth in SEQ ID NO:1 or SEQ ID NO:2 and wherein said variant exhibits decreased sensitivity to ETV and/or LMV and optionally other nucleoside analogs.

10. The isolated HBV variant of Claim 9 wherein said variant exhibits decreased sensitivity to ETV.

11. The isolated HBV variant of Claim 9 wherein said variant exhibits decreased sensitivity to both ETV and LMV.

12. The isolated HBV variant of Claim 9 wherein an antibody specific for a wild-type HBsAg exhibits a reduced capacity to neutralize said HBV variant and wherein said HBV variant is selected by exposure of a subject to ETV and LMV in combination or sequential therapy.

13. The isolated HBV variant of Claim 1 or 9 comprising a mutation in the HBV DNA polymerase selected from spacerL97I, spacerK115R, spacerH116L, spacerL128F, spacerS137G, spacerR139G, spacerF142S, rtY54H, rtL91I, rtA97V, rtY124H, rtH126R, rtS135Y, rtI169T, rtV173L, rtL180M, rtM204V, rtA21S, rtA38E, rtF122L, rtT128N, rtQ130P, rtT184G, rtS202I, rtH248N, rtY252L or a combination thereof or an equivalent mutation.

14. The isolated HBV variant of Claim 1 or 9 or 13 comprising a mutation selected from PreS1 N114D, PreS1 T115S, PreS2 F22L, PreS2 V39A, PreS2 P52L, sL89V, sT118A, sI61L, sE164D, sI195M, sI208T PreS1 E86Q, PreS1 N91K, PreS2 P41H, sQ30K, sP120T,



sL176V, sV194F or a combination thereof or an equivalent mutation.

15. The isolated HBV variant of Claim 1 or 9 or 13 comprising a mutation selected from sF161L, sE164D and sI195M or a combination thereof or an equivalent mutation.

16. The isolated HBV variant of Claim 1 or 9 or 13 comprising a mutation selected from rI169T, rV173L, rL180M, rT184G, rS202I and rM204V or a combination thereof or an equivalent mutation.

17. A method for determining the potential for an HBV to exhibit reduced sensitivity to ETV and/or LMV or optionally other nucleoside analogs, said method comprising isolating DNA or corresponding mRNA from said HBV and screening for a mutation in the nucleotide sequence encoding HBV DNA polymerase resulting in at least one amino acid substitution, deletion and/or addition in any one or more of domains F and A through E or a region proximal thereto of said DNA polymerase and associated with resistance or decreased sensitivity to ETV and/or LMV wherein the presence of such a mutation is an indication of the likelihood of resistance to said ETV and/or LMV.

18. The method of Claim 17 wherein the mutation screened for is selected from spacerL97I, spacerK115R, spacerH116L, spacerL128F, spacerS137G, spacerR139G, spacerF142S, rY54H, rL91I, rA97V, rY124H, rH126R, rS135Y, rI169T, rV173L, rL180M, rM204V, rA21S, rA38E, rF122L, rT128N, rQ130P, rT184G, rS202I, rH248N, rY252L or a combination thereof or an equivalent mutation.

19. A method for determining whether an HBV strain exhibits reduced sensitivity to a nucleoside analog, said method comprising isolating DNA or corresponding mRNA from said HBV and screening for a mutation in the nucleotide sequence encoding the DNA polymerase wherein the presence of the following mutations in the spacer region and the rt region: spacerL97I, spacerK115R, spacerH116L, spacerL128F, spacerS137G, spacerR139G, spacerF142S, rY54H, rL91I, rA97V, rY124H, rH126R, rS135Y, rI169T, rV173L,

rtL180M, rtM204V, rtA21S, rtA38E, rtF122L, rtT128N, rtQ130P, rtT184G, rtS202I, rtH248N, rtY252L or combinations thereof or an equivalent one or more other mutation is indicative of a variant wherein said variant exhibits a decreased sensitivity to ETV and/or LMV and optionally other nucleoside analogs.

20. The method of Claim 19 wherein the mutation screened for is selected from rtI169T, rtV173, rtL180M, rtT184G, rtS202I and rtM204V or a combination thereof or an equivalent mutation.

21. A method for determining whether an HBV strain exhibits reduced sensitivity to a nucleoside analog, said method comprising isolating DNA or corresponding mRNA from said HBV and screening for a mutation in the nucleotide sequence encoding the DNA polymerase wherein the presence of the following mutations in the B or C domain of the rt region: rtI169T, rtV173L, rtL180M, rtT184G, rtS202I, rtM204V or combinations thereof or an equivalent one or more other mutation is indicative of a variant wherein said variant exhibits a decreased sensitivity to ETV and/or LMV and optionally other nucleoside analogs.

22. A method for detecting an agent which exhibits inhibitory activity to an HBV, exhibiting resistance or decreased sensitivity to ETV and/or LMV, said method comprising:

generating a genetic construct comprising a replication competent-effective amount of the genome from said HBV contained in a plasmid vector and then transfecting said cells with said construct;

contacting said cells, before, during and/or after transfection, with the agent to be tested;

culturing said cells for a time and under conditions sufficient for the HBV to replicate, express genetic sequences and/or assemble and/or release virus or virus-like particles if resistant to said agent; and

subjecting the cells, cell lysates or culture supernatant fluid to viral- or viral-component-detection means to determine whether or not the virus has replicated, expressed genetic material and/or assembled and/or been released in the presence of said agent.

23. A method for detecting an agent which exhibits inhibitory activity to an HBV, exhibiting resistance or decreased sensitivity to ETV and/or LMV, said method comprising:

generating a genetic construct comprising a replication competent-effective amount of the genome from said HBV contained in or fused to an amount of a baculovirus genome effective to infect cells and then infecting said cells with said construct;

contacting said cells, before, during and/or after infection, with the agent to be tested;

culturing said cells for a time and under conditions sufficient for the HBV to replicate, express genetic sequences and/or assemble and/or release virus or virus-like particles if resistant to said agent; and

subjecting the cells, cell lysates or culture supernatant fluid to viral- or viral-component-detection means to determine whether or not the virus has replicated, expressed genetic material and/or assembled and/or been released in the presence of said agent.

24. A method for detecting an agent which exhibits inhibitory activity to an HBV, exhibiting resistance or decreased sensitivity to ETV and/or LMV, said method comprising:

generating a genetic construct comprising a replication competent-effective amount of the genome from said HBV contained in or fused to an amount of a baculovirus genome effective to infect cells and then infecting said cells with said construct;

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contacting said cells, before, during and/or after infection, with the agent to be tested;

culturing said cells for a time and under conditions sufficient for the HBV to replicate, express genetic sequences and/or assemble and/or release virus or virus-like particles if resistant to said agent; and

subjecting the cells, cell lysates or culture supernatant fluid to viral- or viral-component-detection means to determine whether or not the virus has replicated, expressed genetic material and/or assembled and/or been released in the presence of said agent.

25. The method of Claims 22 or 23 or 24 wherein the HBV genome is stably integrated into the cells' genome.
26. A vaccine comprising an antigenic component of the HBV variant of any one of Claims 1 to 16 or an antibody thereto.
27. The vaccine of Claim 26 wherein the antigenic component is an HBsAg or PreS1 or PreS2.
28. The vaccine of Claim 26 wherein the antigenic component is a defective HBV variant.
29. The vaccine of Claim 26 comprising an antibody to HBsAg or PreS1 or PreS2.
30. A computer product for assessing the likely usefulness of a viral variant or biological sample comprising same for determining an appropriate therapeutic protocol in a subject, said product comprising:

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- (1) code that receives as input  $I_V$ s for at least two features associated with said viral agents or biological sample comprising same, wherein said features are selected from:
  - (f) the ability to exhibit resistance for reduced sensitivity to a particular compound or immunological agent;
  - (g) an altered DNA polymerase from wild-type HBV;
  - (h) an altered surface antigen from wild-type HBV; or
  - (i) morbidity or recovery potential of a patient;
  - (j) altered replication capacity (increased or decreased);
- (2) code that adds said  $I_V$ s to provide a sum corresponding to a  $P_V$  for said viral variants or biological samples; and
- (3) a computer readable medium that stores the codes.

31. A computer for assessing the likely usefulness of a viral variant or biological sample comprising same in a subject, wherein said computer comprises:

- (1) a machine-readable data storage medium comprising a data storage material encoded with machine-readable data, wherein said machine-readable data comprise  $I_V$ s for at least two features associated with said viral variant or biological sample; wherein said features are selected from:-
  - (a) the ability to exhibit resistance for reduced sensitivity to a particular compound or immunological agent;
  - (b) an altered DNA polymerase from wild-type HBV;
  - (c) an altered surface antigen from wild-type HBV; or
  - (d) morbidity or recovery potential of a patient;

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- (e) altered replication capacity (increased or decreased);
  - (2) a working memory for storing instructions for processing said machine-readable data;
  - (3) a central-processing unit coupled to said working memory and to said machine-readable data storage medium, for processing said machine readable data to provide a sum of said  $I_{VS}$  corresponding to a  $P_V$  for said compound(s); and
  - (4) an output hardware coupled to said central processing unit, for receiving said  $P_V$ .
32. A composition comprising an agent capable of directly or indirectly inhibiting an HBV variant as defined in any one of Claims 1 to 16, said composition further comprising one or more pharmaceutically acceptable carriers and/or diluents.
33. The composition of Claim 32 wherein the agent is a recombinant protein from said HBV variant.
34. The composition of Claim 33 wherein the recombinant protein is HBsAg or PreS1 or PreS2.
35. The composition of Claim 32 wherein the agent is capable of inhibiting an HBV variant polymerase.
36. The composition of Claim 35 wherein the agent is identified by natural product screening or rational drug design.
37. The composition of Claim 32 wherein the agent is a defective HBV variant.

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38. The composition of Claim 32 wherein the agent is an antibody directed to an HBV compound.

39. The composition of Claim 32 wherein the agent is a ribozyme, antisense molecule or sense molecule relative to an HBV gene.

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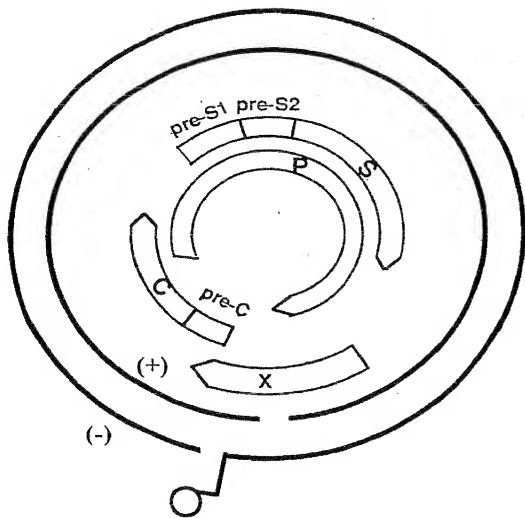


Figure 1



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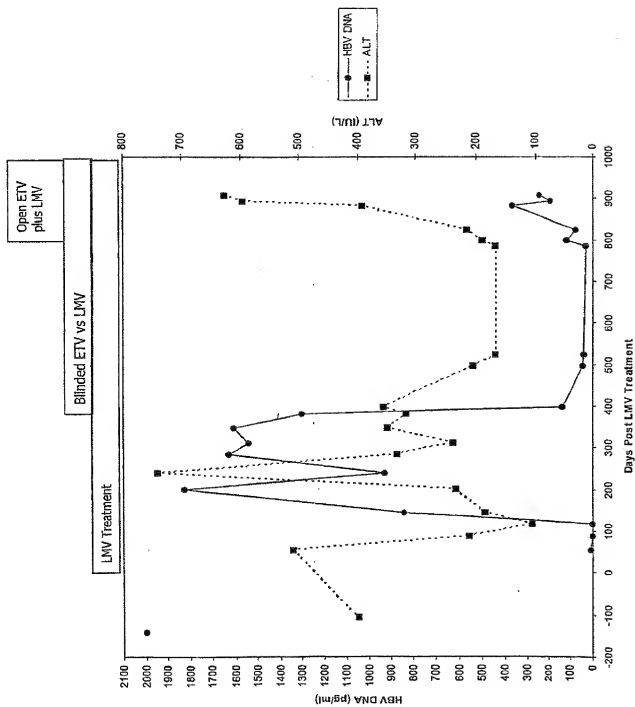
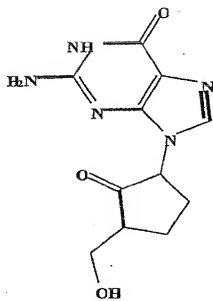


Figure 2

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**Figure 3**

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### Figure 4







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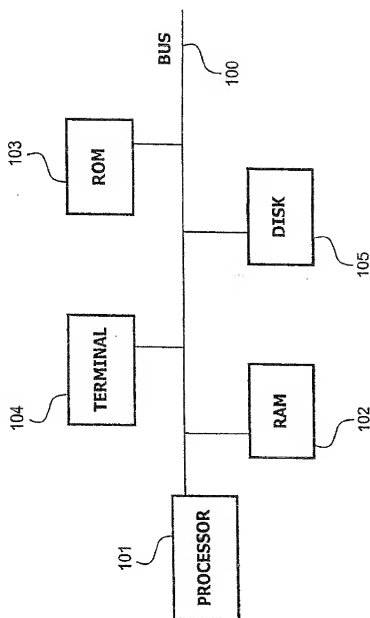


Figure 7





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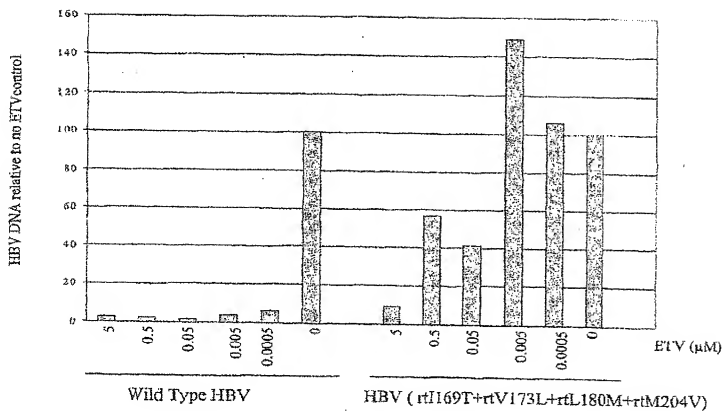
Figure 9

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Post ETV	1			[SEQ ID NO:36]
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		NEMTSQFLGRLNLQNGFFILTRILITIPKSLDGNWNTSLNFIAGTIVCLGQNSQPTS		
			110	
Pre ETV	101	NHSPTSCPP*		[SEQ ID NO:35]
Post ETV	59	NHSPTSCPPCGRYEMCLERFIIFILLCLIFILNLDYQMLPVCELISSSTISGTGTCVTPAQTSWNPSCCTKFSQNCVCIPISSMAF	158	[SEQ ID NO:36]
			110	
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Figure 10

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**Figure 11**

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Southern Health (all states except US)  
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Locarnini, Stephen (US only)  
Ayres, Anna (US only)  
Angus, Peter (US only)  
Sievvert, William (US only)

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<210> 16  
 <211> 614  
 <212> DNA  
 <213> TR4

- 15 -

<400> 16  
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 cgactactgc tcaaggaacc tctatgtatc cctcctgttg ctgtacacaa ccttcggacg 240  
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 tgtacagcac cttgagtcce tttttaccgc tgttaccat tttcttttgt ctttgggtat 480  
 acatttaaac cctaacacaaa ctaaaagatg ggggtattcc ttaaatttca tgggctatgt 540  
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 taggaaactt cctg 614

<210> 17  
 <211> 607  
 <212> DNA  
 <213> TR5

<400> 17  
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 cgactactgc tcaaggaacc tctatgtatc cctcctgttg ctgtacacaa ccttcggacg 240  
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 ggctttcccc cactgtttgg ctttcagtta tgtggatgat gtggatttgg gggccaagtc 420  
 tgtacagcac cttgagtcce tttttaccgc tgttaccat tttcttttgt ctttgggtat 480  
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 taggaaa 607

<210> 18  
 <211> 1028

- 16 -

&lt;212&gt; DNA

&lt;213&gt; TR6

&lt;220&gt;

&lt;221&gt; misc\_feature

&lt;222&gt; (12)..(12)

&lt;223&gt; N = any nucleotide

&lt;220&gt;

&lt;221&gt; misc\_feature

&lt;222&gt; (27)..(27)

&lt;223&gt; N = any nucleotide

&lt;400&gt; 18

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&lt;210&gt; 19

&lt;211&gt; 181

- 17 -

&lt;212&gt; PRT

&lt;213&gt; Pol Trans of TR1

&lt;220&gt;

&lt;221&gt; misc\_feature

&lt;222&gt; (130)..(130)

&lt;223&gt; X = any amino acid

&lt;400&gt; 19

Ser Asn Leu Ser Trp Leu Ser Leu Asp Val Ser Ala Ala Phe Tyr His  
 1 5 10 15

Ile Pro Leu His Pro Ala Ala Met Pro His Leu Leu Val Gly Ser Ser  
 20 25 30

Gly Leu Ser Arg Tyr Val Ala Arg Leu Ser Ser Asn Ser Arg Ile Phe  
 35 40 45

Asn His Gln Arg Gly Thr Met Gln Asn Leu His Asp Tyr Cys Ser Arg  
 50 55 60

Asn Leu Tyr Val Ser Leu Leu Leu Tyr Gln Thr Phe Gly Arg Lys  
 65 70 75 80

Leu His Leu Tyr Ser His Pro Ile Ile Leu Gly Phe Arg Lys Ile Pro  
 85 90 95

Met Gly Val Gly Leu Ser Pro Phe Leu Met Ala Gln Phe Thr Ser Ala  
 100 105 110

Ile Cys Ser Val Val Arg Arg Ala Phe Pro His Cys Leu Ala Phe Ser  
 115 120 125

Tyr Xaa Asp Asp Val Val Leu Gly Ala Lys Ser Val Gln His Leu Glu  
 130 135 140

Ser Leu Phe Thr Ala Val Thr Asn Phe Leu Leu Ser Leu Gly Ile His  
 145 150 155 160

Leu Asn Pro Asn Lys Thr Lys Arg Trp Gly Tyr Ser Leu Asn Phe Met  
 165 170 175

- 18 -

Gly Tyr Val Ile Gly  
180

<210> 20  
<211> 187  
<212> PRT  
<213> Pol Trans of TR2

<400> 20

Leu Ser Ser Asn Leu Ser Trp Leu Ser Leu Asp Val Ser Ala Ala Phe  
1 5 10 15

Tyr His Ile Pro Leu His Pro Ala Ala Met Pro His Leu Leu Val Gly  
20 25 30

Ser Ser Gly Leu Ser Arg Tyr Val Ala Arg Leu Ser Ser Asn Ser Arg  
35 40 45

Ile Phe Asn His Gln Arg Gly Thr Met Gln Asn Leu His Asp Tyr Cys  
50 55 60

Ser Arg Asn Leu Tyr Val Ser Leu Leu Leu Tyr Gln Thr Phe Gly  
65 70 75 80

Arg Lys Leu His Leu Tyr Ser His Pro Ile Ile Leu Gly Phe Arg Lys  
85 90 95

Ile Pro Met Gly Val Gly Leu Ser Pro Phe Leu Met Ala Gln Phe Thr  
100 105 110

Ser Ala Ile Cys Ser Val Val Arg Arg Ala Phe Pro His Cys Leu Ala  
115 120 125

Phe Ser Tyr Val Asp Asp Val Val Leu Gly Ala Lys Ser Val Gln His  
130 135 140

Leu Glu Ser Leu Phe Thr Ala Val Thr Asn Phe Leu Leu Ser Leu Gly  
145 150 155 160

Ile His Leu Asn Pro Asn Lys Thr Lys Arg Trp Gly Tyr Ser Leu Asn  
165 170 175

- 19 -

Phe Met Gly Tyr Val Ile Gly Cys Tyr Gly Ser  
180 185

<210> 21

<211> 185

<212> PRT

<213> Pol Trans of TR3

<400> 21

Leu Ser Ser Asn Leu Ser Trp Leu Ser Leu Asp Val Ser Ala Ala Phe  
1 5 10 15

Tyr His Ile Pro Leu His Pro Ala Ala Met Pro His Leu Leu Val Gly  
20 25 30

Ser Ser Gly Leu Ser Arg Tyr Val Ala Arg Leu Ser Ser Asn Ser Arg  
35 40 45

Ile Phe Asn His Gln Arg Gly Thr Met Gln Asn Leu His Asp Tyr Cys  
50 55 60

Ser Arg Asn Leu Tyr Val Ser Leu Leu Leu Tyr Gln Thr Phe Gly  
65 70 75 80

Arg Lys Leu His Leu Tyr Ser His Pro Ile Ile Leu Gly Phe Arg Lys  
85 90 95

Ile Pro Met Gly Val Gly Leu Ser Pro Phe Leu Met Ala Gln Phe Thr  
100 105 110

Ser Ala Ile Cys Ser Val Val Arg Arg Ala Phe Pro His Cys Leu Ala  
115 120 125

Phe Ser Tyr Val Asp Asp Val Val Leu Gly Ala Lys Ser Val Gln His  
130 135 140

Leu Glu Ser Leu Phe Thr Ala Val Thr Asn Phe Leu Leu Ser Leu Gly  
145 150 155 160

Ile His Leu Asn Pro Asn Lys Thr Lys Arg Trp Gly Tyr Ser Leu Asn  
165 170 175

- 20 -

Phe Met Gly Tyr Val Ile Gly Cys Tyr  
180 185

<210> 22  
<211> 184  
<212> PRT  
<213> Pol Trans of TR4

<400> 22

Ser Ser Asn Leu Ser Trp Leu Ser Leu Asp Val Ser Ala Ala Phe Tyr  
1 5 10 15

His Ile Pro Leu His Pro Ala Ala Met Pro His Leu Leu Val Gly Ser  
20 25 30

Ser Gly Leu Ser Arg Tyr Val Ala Arg Leu Ser Ser Asn Ser Arg Ile  
35 40 45

Phe Asn His Gln Arg Gly Thr Met Gln Asn Leu His Asp Tyr Cys Ser  
50 55 60

Arg Asn Leu Tyr Val Ser Leu Leu Leu Tyr Gln Thr Phe Gly Arg  
65 70 75 80

Lys Leu His Leu Tyr Ser His Pro Ile Ile Leu Gly Phe Arg Lys Ile  
85 90 95

Pro Met Gly Val Gly Leu Ser Pro Phe Leu Met Ala Gln Phe Thr Ser  
100 105 110

Ala Ile Cys Ser Val Val Arg Arg Ala Phe Pro His Cys Leu Ala Phe  
115 120 125

Ser Tyr Val Asp Asp Val Val Leu Gly Ala Lys Ser Val Gln His Leu  
130 135 140

Glu Ser Leu Phe Thr Ala Val Thr Asn Phe Leu Leu Ser Leu Gly Ile  
145 150 155 160

His Leu Asn Pro Asn Lys Thr Lys Arg Trp Gly Tyr Ser Leu Asn Phe  
165 170 175

- 21 -

Met Gly Tyr Val Ile Gly Cys Tyr  
180

<210> 23  
<211> 184  
<212> PRT  
<213> Pol Trans of TR5

<220>  
<221> misc\_feature  
<222> (100)..(100)  
<223> X = any amino acid

<400> 23

Ser Ser Asn Leu Ser Trp Leu Ser Leu Asp Val Ser Ala Ala Phe Tyr  
1 5 10 15

His Ile Pro Leu His Pro Ala Ala Met Pro His Leu Leu Val Gly Ser  
20 25 30

Ser Gly Leu Ser Arg Tyr Val Ala Arg Leu Ser Ser Asn Ser Arg Ile  
35 40 45

Phe Asn His Gln Arg Gly Thr Met Gln Asn Leu His Asp Tyr Cys Ser  
50 55 60

Arg Asn Leu Tyr Val Ser Leu Leu Leu Leu Tyr Gln Thr Phe Gly Arg  
65 70 75 80

Lys Leu His Leu Tyr Ser His Pro Ile Ile Leu Gly Phe Arg Lys Ile  
85 90 95

Pro Met Gly Xaa Gly Leu Ser Pro Phe Leu Met Ala Gln Phe Thr Ser  
100 105 110

Ala Ile Cys Ser Val Val Arg Arg Ala Phe Pro His Cys Leu Ala Phe  
115 120 125

Ser Tyr Val Asp Asp Val Val Leu Gly Ala Lys Ser Val Gln His Leu  
130 135 140

Glu Ser Leu Phe Thr Ala Val Thr Asn Phe Leu Leu Ser Leu Gly Ile  
145 150 155 160



- 22 -

His Leu Asn Pro Asn Lys Thr Lys Arg Trp Gly Tyr Ser Leu Asn Phe  
 165 170 175

Met Gly Tyr Val Ile Gly Cys Tyr  
 180

<210> 24  
 <211> 326  
 <212> PRT  
 <213> Pol Trans of TR6

<220>  
 <221> misc feature  
 <222> (168)..(168)  
 <223> X = any amino acid

<400> 24

Ile Tyr Gln Ser Pro Val Arg Lys Ala Ala Tyr Pro Ala Val Ser Thr  
 1 5 10 15

Phe Glu Arg Leu Ser Ser Ser Gly His Ala Val Glu Leu His Asn Phe  
 20 25 30

Pro Pro Asn Ser Ala Arg Ser Gln Gly Glu Gly Pro Val Ser Pro Cys  
 35 40 45

Trp Trp Leu Gln Phe Arg Asn Ser Lys Pro Cys Ser Asp Tyr Cys Leu  
 50 55 60

Ser His Ile Val Asn Leu Leu Glu Asp Trp Gly Pro Cys Ala Glu His  
 65 70 75 80

Gly Glu His His Ile Arg Ile Pro Arg Thr Pro Ala Arg Val Thr Gly  
 85 90 95

Gly Val Phe Leu Val Asp Lys Asn Pro His Asn Thr Ala Glu Ser Arg  
 100 105 110

Leu Val Val Asp Phe Ser Gln Phe Ser Arg Gly Asn His Arg Val Ser  
 115 120 125

- 23 -

Trp Pro Lys Phe Ala Val Pro Asn Leu Gln Ser Leu Thr Asn Leu Leu  
 130 135 140

Ser Ser Asn Leu Ser Trp Leu Ser Leu Asp Val Ser Ala Ala Phe Tyr  
 145 150 155 160

His Ile Pro Leu His Pro Ala Xaa Met Pro His Leu Leu Val Gly Ser  
 165 170 175

Ser Gly Leu Ser Arg Tyr Val Ala Arg Leu Ser Ser Asn Ser Arg Ile  
 180 185 190

Phe Asn His Gln Arg Gly Thr Met Gln Asn Leu His Asp Tyr Cys Ser  
 195 200 205

Arg Asn Leu Tyr Val Ser Leu Leu Leu Leu Tyr Gln Thr Phe Gly Arg  
 210 215 220

Lys Leu His Leu Tyr Ser His Pro Ile Ile Leu Gly Phe Arg Lys Thr  
 225 230 235 240

Pro Met Gly Leu Gly Leu Ser Pro Phe Leu Met Ala Gln Phe Thr Ser  
 245 250 255

Ala Ile Cys Ser Val Val Arg Arg Ala Phe Pro His Cys Leu Ala Phe  
 260 265 270

Ser Tyr Val Asp Asp Val Val Leu Gly Ala Lys Ser Val Gln His Leu  
 275 280 285

Glu Ser Leu Phe Thr Ala Val Thr Asn Phe Leu Leu Ser Leu Gly Ile  
 290 295 300

His Leu Asn Pro Asn Lys Thr Lys Arg Trp Gly Tyr Ser Leu Asn Phe  
 305 310 315 320

Val Gly Tyr Val Ile Gly  
 325

<210> 25  
 <211> 161  
 <212> PRT

- 24 -

&lt;213&gt; HBsAg Trans of TR1

&lt;220&gt;

&lt;221&gt; misc\_feature

&lt;222&gt; (111)..(111)

&lt;223&gt; X = any amino acid

&lt;220&gt;

&lt;221&gt; misc\_feature

&lt;222&gt; (129)..(129)

&lt;223&gt; X = any amino acid

&lt;400&gt; 25

Pro	Thr	Cys	Pro	Gly	Tyr	Arg	Trp	Met	Cys	Leu	Arg	Arg	Phe	Ile	Ile
1			5						10					15	

Phe	Leu	Phe	Ile	Leu	Leu	Leu	Cys	Leu	Ile	Phe	Leu	Leu	Val	Leu	Leu
	20							25					30		

Asp	Tyr	Gln	Gly	Met	Leu	Pro	Val	Cys	Pro	Leu	Ile	Pro	Gly	Ser	Ser
	35						40					45			

Thr	Thr	Ser	Ala	Gly	Pro	Cys	Arg	Thr	Cys	Thr	Thr	Thr	Ala	Gln	Gly
	50					55					60				

Thr	Ser	Met	Tyr	Pro	Ser	Cys	Cys	Cys	Thr	Lys	Pro	Ser	Asp	Gly	Asn
65					70					75					80

Cys	Thr	Cys	Ile	Pro	Ile	Pro	Ser	Ser	Trp	Ala	Phe	Gly	Lys	Phe	Leu
			85						90					95	

Trp	Glu	Trp	Ala	Ser	Ala	Arg	Phe	Ser	Trp	Leu	Ser	Leu	Leu	Xaa	Pro
			100					105						110	

Phe	Val	Gln	Trp	Phe	Val	Gly	Leu	Ser	Pro	Thr	Val	Trp	Leu	Leu	Val
		115					120						125		

Xaa	Trp	Met	Met	Trp	Tyr	Trp	Gly	Pro	Ser	Leu	Tyr	Ser	Thr	Leu	Ser
	130					135					140				

Pro	Phe	Leu	Pro	Leu	Leu	Pro	Ile	Phe	Phe	Cys	Leu	Trp	Val	Tyr	Ile
145						150				155					160

- 25 -

Asn

<210> 26  
 <211> 162  
 <212> PRT  
 <213> HBSAg Trans of TR2  
  
 <220>  
 <221> misc\_feature  
 <222> (113)..(113)  
 <223> X = any amino acid

&lt;400&gt; 26

Cys Pro Pro Thr Cys Pro Gly Tyr Arg Trp Met Cys Leu Arg Arg Phe  
 1 5 10 15

Ile Ile Phe Leu Phe Ile Leu Leu Leu Cys Leu Ile Phe Leu Leu Val  
 20 25 30

Leu Leu Asp Tyr Gln Gly Met Leu Pro Val Cys Pro Leu Ile Pro Gly  
 35 40 45

Ser Ser Thr Thr Ser Ala Gly Pro Cys Arg Thr Cys Thr Thr Thr Ala  
 50 55 60

Gln Gly Thr Ser Met Tyr Pro Ser Cys Cys Cys Thr Lys Pro Ser Asp  
 65 70 75 80

Gly Asn Cys Thr Cys Ile Pro Ile Pro Ser Ser Trp Ala Phe Gly Lys  
 85 90 95

Phe Leu Trp Glu Trp Ala Ser Ala Arg Phe Ser Trp Leu Ser Leu Leu  
 100 105 110

Xaa Pro Phe Val Gln Trp Phe Val Gly Leu Ser Pro Thr Val Trp Leu  
 115 120 125

Ser Val Met Trp Met Met Trp Tyr Trp Gly Pro Ser Leu Tyr Ser Thr  
 130 135 140

Leu Ser Pro Phe Leu Pro Leu Leu Pro Ile Phe Phe Cys Leu Trp Val

- 26 -

145                      150                      155                      160

Tyr Ile

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<210> 27
<211> 162
<212> PRT
<213> HBsAg Trans of TR3
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<220>
<221> misc_feature .
<222> {129}..{129}
<223> X = any amino acid
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<400> 27

Cys Pro Pro Thr Cys Pro Gly Tyr Arg Trp Met Cys Leu Arg Arg Phe  
1 5 10 15

Ile Ile Phe Leu Phe Ile Leu Leu Leu Cys Leu Ile Phe Leu Leu Val  
20 25 30

Leu Leu Asp Tyr Gln Gly Met Leu Pro Val Cys Pro Leu Ile Pro Gly  
35 40 45

Ser Ser Thr Thr Ser Ala Gly Pro Cys Arg Thr Cys Thr Thr Thr Ala  
50 55 60

Gln Gly Thr Ser Met Tyr Pro Ser Cys Cys Cys Thr Lys Pro Ser Asp  
65                      70                      75                      80

Gly Asn Cys Thr Cys Ile Pro Ile Pro Ser Ser Trp Ala Phe Gly Lys  
85 90 95

Phe Leu Trp Glu Trp Ala Ser Ala Arg Phe Ser Trp Leu Ser Leu Leu  
100 105 110

Val Pro Phe Val Gln Trp Phe Val Gly Leu Ser Pro Thr Val Trp Leu  
115 120 125

Xaa Val Met Trp Met Met Trp Tyr Trp Gly Pro Ser Leu Tyr Ser Thr  
130 135 140

- 27 -

Leu Ser Pro Phe Leu Pro Leu Leu Pro Ile Phe Phe Cys Leu Trp Val  
 145 150 155 160

Tyr Ile

<210> 28  
 <211> 161  
 <212> PRT  
 <213> HBsAg Trans of TR4  
 <400> 28

Pro Pro Thr Cys Pro Gly Tyr Arg Trp Met Cys Leu Arg Arg Phe Ile  
 1 5 10 15

Ile Phe Leu Phe Ile Leu Leu Leu Cys Leu Ile Phe Leu Leu Val Leu  
 20 25 30

Leu Asp Tyr Gln Gly Met Leu Pro Val Cys Pro Leu Ile Pro Gly Ser  
 35 40 45

Ser Thr Thr Ser Ala Gly Pro Cys Arg Thr Cys Thr Thr Thr Ala Gln  
 50 55 60

Gly Thr Ser Met Tyr Pro Ser Cys Cys Cys Thr Lys Pro Ser Asp Gly  
 65 70 75 80

Asn Cys Thr Cys Ile Pro Ile Pro Ser Ser Trp Ala Phe Gly Lys Phe  
 85 90 95

Leu Trp Glu Trp Ala Ser Ala Arg Phe Ser Trp Leu Ser Leu Leu Val  
 100 105 110

Pro Phe Val Gln Trp Phe Val Gly Leu Ser Pro Thr Val Trp Leu Ser  
 115 120 125

Val Met Trp Met Met Trp Tyr Trp Gly Pro Ser Leu Tyr Ser Thr Leu  
 130 135 140

Ser Pro Phe Leu Pro Leu Leu Pro Ile Phe Phe Cys Leu Trp Val Tyr  
 145 150 155 160

- 28 -

Ile

<210> 29  
 <211> 161  
 <212> PRT  
 <213> HBSAg Trans of TR5

<220>  
 <221> misc\_feature  
 <222> (99)..(99)  
 <223> X = any amino acid

&lt;400&gt; 29

Pro Pro Thr Cys Pro Gly Tyr Arg Trp Met Cys Leu Arg Arg Phe Ile  
 1 5 10 15

Ile Phe Leu Phe Ile Leu Leu Leu Cys Leu Ile Phe Leu Leu Val Leu  
 20 25 30

Leu Asp Tyr Gln Gly Met Leu Pro Val Cys Pro Leu Ile Pro Gly Ser  
 35 40 45

Ser Thr Thr Ser Ala Gly Pro Cys Arg Thr Cys Thr Thr Thr Ala Gln  
 50 55 60

Gly Thr Ser Met Tyr Pro Ser Cys Cys Cys Thr Lys Pro Ser Asp Gly  
 65 70 75 80

Asn Cys Thr Cys Ile Pro Ile Pro Ser Ser Trp Ala Phe Gly Lys Phe  
 85 90 95

Leu Trp Xaa Trp Ala Ser Ala Arg Phe Ser Trp Leu Ser Leu Leu Val  
 100 105 110

Pro Phe Val Gln Trp Phe Val Gly Leu Ser Pro Thr Val Trp Leu Ser  
 115 120 125

Val Met Trp Met Met Trp Tyr Trp Gly Pro Ser Leu Tyr Ser Thr Leu  
 130 135 140

Ser Pro Phe Leu Pro Leu Leu Pro Ile Phe Phe Cys Leu Trp Val Tyr

- 29 -

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145                               150                               155                               160

Ile

<210> 30
<211> 305
<212> PRT
<213> HBSAg Trans of TR5

<220>
<221> misc_feature
<222> (168)..(168)
<223> X = any amino acid

<400> 30

Ser Thr Asn Arg Gln Ser Gly Arg Gln Pro Thr Pro Leu Ser Pro Pro
1          5          10

Leu Arg Asp Ser His Pro Gln Ala Met Gln Trp Asn Ser Thr Thr Phe
          20          25          30

His Gln Thr Leu Gln Asp Pro Arg Val Arg Gly Leu Tyr Leu Pro Ala
          35          40          45

Gly Gly Ser Ser Ser Gly Thr Val Asn Pro Val Pro Thr Thr Ala Ser
          50          55          60

Pro Ile Ser Ser Ile Phe Ser Arg Ile Gly Asp Leu Ala Leu Asn Met
65          70          75          80

Glu Asn Ile Thr Ser Gly Phe Leu Gly Pro Leu Leu Val Leu Gln Ala
          85          90          95

Gly Phe Phe Leu Leu Thr Arg Ile Leu Thr Ile Pro Gln Ser Leu Asp
          100          105          110

Ser Trp Trp Thr Ser Leu Asn Phe Leu Gly Gly Thr Thr Val Cys Leu
          115          120          125

Gly Gln Asn Ser Gln Ser Pro Thr Ser Asn His Ser Pro Thr Ser Cys
          130          135          140

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Pro Pro Thr Cys Pro Gly Tyr Arg Trp Met Cys Leu Arg Arg Phe Ile  
145 150 155 160

Ile Phe Leu Phe Ile Leu Leu Xaa Cys Leu Ile Phe Leu Leu Val Leu  
165 170 175

Leu Asp Tyr Gln Gly Met Leu Pro Val Cys Pro Leu Ile Pro Gly Ser  
180 185 190

Ser Thr Thr Ser Ala Gly Pro Cys Arg Thr Cys Thr Thr Thr Ala Gln  
195 200 205

Gly Thr Ser Met Tyr Pro Ser Cys Cys Cys Thr Lys Pro Ser Asp Gly  
210 215 220

Asn Cys Thr Cys Ile Pro Ile Pro Ser Ser Trp Ala Phe Gly Lys Leu  
225 230 235 240

Leu Trp Asp Trp Ala Ser Ala Arg Phe Ser Trp Leu Ser Leu Leu Val  
245 250 255

Pro Phe Val Gln Trp Phe Val Gly Leu Ser Pro Thr Val Trp Leu Ser  
260 265 270

Val Met Trp Met Met Trp Tyr Trp Gly Pro Ser Leu Tyr Ser Thr Leu  
275 280 285

Ser Pro Phe Leu Pro Leu Leu Pro Ile Phe Phe Cys Leu Trp Val Tyr  
290 295 300

Ile  
305

<210> 31  
<211> 945  
<212> DNA  
<213> pre ETV

<400> 31  
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gtaaaccctg ttccgactac tgcctctcac atatgtcaa tcttctcgag gattggggac 120

- 31 -

cctgcgctga atatggagaa catcacatca ggattcctag gaccccttct cgtgttacag	180
gcggggtttt tcttgttgac aagaatcctc acaataccgm agagtctaga ctogtgggtgg	240
acttctctca attttctagg gggaaccacc gtgtgtcttg gccaaaatc gcagtcacca	300
acctccaate actcaccaac ctctgtcct cgcacttgac ctggttatcg ctggatgtgt	360
ctgcggcggt ttatcatatt cctcttcate ctgctgctat gcctcatctt cttgttggtt	420
cttctggact atcaagggtat gttgcccggt tgcctctaa ttccaggatc ctcaaccacc	480
agcacgggaa catgcogaac ttgcacgact cctgctcaag gaacctctat gtatccctcc	540
tgttgctgta ccaaaccctc ggacggaaat tgcacctgta ttcccatccc atcatcctgg	600
gctttcgga aattcctatg ggagtgggac tcagcccggt tctcatggct cagtttasta	660
gtgccatttg ttcagtgtt cgtagggtt tccccactg tttggcttcc agttatgtgg	720
atgatgtggt attgggggac aagtctgtac agcatcttga gtcccttttt accgctgtta	780
ccaattttct tttgtctctg ggtatacatt tgaaccctaa caaacaag agatgggggtt	840
actccctaaa ttttatgggc tatgtcattg gatgttatgg gtccctggca caagaacaca	900
tcgtacataa aatcaaagaa tgttttagaa aacttcctgt taaca	945

<210> 32  
 <211> 1245  
 <212> DNA  
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caaacacccg aaatccagat tgggacttca atcccaacaa ggacacctgg ccagacgcga	180
acaaagtagg agctggagca ttccgggtcg gtttcacccc accgcacgga ggccttttgg	240
ggtggagccc tcaggctcag ggcatactac aaactttgac agcaaaagcc cctcctgcct	300
ccaccaatcg ccagtcagga cggcagccta ccccgctgtc tccaccttgg agagacactc	360
atcctcaggg gcagtggaaa cccacaacct tccaccaaac tgtgcaagct ccacctgtgt	420
gtggctccag ttccggaaca gtaaaccttg ttccgactac tgccctctac atatcgtaaa	480
tcttctcgag gattggggac cctgcgctga atatggagaa catcacatca ggattcctag	540
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- 32 -

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agagctctaga ctcggtggtgg acttctctca atttctctagg gggaaccacc gtgtgtcttg 660
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ctgggttatcg ctggatgtgt ctgcccgtt ttatcatatt cctcttcate ctgctgctat 780
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Phe Arg Asn Ser Lys Pro Cys Ser Asp Tyr Cys Leu Ser His Ile Val
          20          25          30

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- 33 -

Asn Leu Leu Glu Asp Trp Gly Pro Cys Ala Glu Tyr Gly Glu His His  
35 40 45

Ile Arg Ile Pro Arg Thr Pro Ser Arg Val Thr Gly Gly Val Phe Leu  
50 55 60

Val Asp Lys Asn Pro His Asn Thr Xaa Glu Ser Arg Leu Val Val Asp  
65 70 75 80

Phe Ser Gln Phe Ser Arg Gly Asn His Arg Val Ser Trp Pro Lys Phe  
85 90 95

Ala Val Pro Asn Leu Gln Ser Leu Thr Asn Leu Leu Ser Ser Asp Leu  
100 105 110

Thr Trp Leu Ser Leu Asp Val Ser Ala Ala Phe Tyr His Ile Pro Leu  
115 120 125

His Pro Ala Ala Met Pro His Leu Leu Val Gly Ser Ser Gly Leu Ser  
130 135 140

Arg Tyr Val Ala Arg Leu Ser Ser Asn Ser Arg Ile Leu Asn His Gln  
145 150 155 160

His Gly Asn Met Pro Asn Leu His Asp Ser Cys Ser Arg Asn Leu Tyr  
165 170 175

Val Ser Leu Leu Leu Leu Tyr Gln Thr Phe Gly Arg Lys Leu His Leu  
180 185 190

Tyr Ser His Pro Ile Ile Leu Gly Phe Arg Lys Ile Pro Met Gly Val  
195 200 205

Gly Leu Ser Pro Phe Leu Met Ala Gln Phe Xaa Ser Ala Ile Cys Ser  
210 215 220

Val Val Arg Arg Ala Phe Pro His Cys Leu Ala Phe Ser Tyr Val Asp  
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Asp Val Val Leu Gly Ala Lys Ser Val Gln His Leu Glu Ser Leu Phe  
245 250 255

- 34 -

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Ile Gly  
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 35 40 45

Phe Ser Arg Gly Asn His Arg Val Ser Trp Pro Lys Phe Ala Val Pro  
 50 55 60

Asn Leu Gln Ser Leu Thr Asn Leu Leu Ser Ser Asp Leu Ser Trp Leu  
 65 70 75 80

Ser Leu Asp Val Ser Ala Ala Phe Tyr His Ile Pro Leu His Pro Ala  
 85 90 95

Ala Met Pro His Leu Leu Val Gly Ser Ser Gly Leu Ser Arg Tyr Val  
 100 105 110

Ala Arg Leu Ser Ser Asn Ser Arg Ile Leu Asn His Gln His Gly Asn  
 115 120 125

Met Pro Asn Leu His Asp Ser Cys Ser Arg Asn Leu Tyr Val Ser Leu  
 130 135 140

- 35 -

Leu Leu Leu Tyr Gln Thr Phe Gly Arg Lys Leu His Leu Tyr Ser His  
 145 150 155 160

Pro Ile Ile Leu Gly Phe Arg Lys Ile Pro Met Gly Val Gly Leu Ser  
 165 170 175

Pro Phe Leu Met Ala Gln Phe Gly Ser Ala Ile Cys Ser Val Val Arg  
 180 185 190

Arg Ala Phe Pro His Cys Leu Ala Phe Ile Tyr Val Asp Asp Val Val  
 195 200 205

Leu Gly Ala Lys Ser Val Gln His Leu Glu Ser Leu Phe Thr Ala Val  
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- 36 -

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Gly Phe Leu Gly Pro Leu Leu Val Leu Gln Ala Gly Phe Phe Leu Leu  
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Asp Ser Trp Trp Thr Ser Leu Asn Phe Leu Gly Gly Thr Thr Val Cys  
35 40 45

Leu Gly Gln Asn Ser Gln Ser Pro Thr Ser Asn His Ser Pro Thr Ser  
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Cys Pro Pro Thr Cys Pro Gly Tyr Arg Trp Met Cys Leu Arg Arg Phe  
65 70 75 80

Ile Ile Phe Leu Phe Ile Leu Leu Leu Cys Leu Ile Phe Leu Leu Val  
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Leu Leu Asp Tyr Gln Gly Met Leu Pro Val Cys Pro Leu Ile Pro Gly  
100 105 110

- 37 -

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115 120 125

Gln Gly Thr Ser Met Tyr Pro Ser Cys Cys Cys Thr Lys Pro Ser Asp  
130 135 140

Gly Asn Cys Thr Cys Ile Pro Ile Pro Ser Ser Trp Ala Phe Gly Lys  
145 150 155 160

Phe Leu Trp Glu Trp Ala Ser Ala Arg Phe Ser Trp Leu Ser Leu Val  
165 170 175

Val Pro Phe Val Gln Trp Phe Val Gly Leu Ser Pro Thr Val Trp Leu  
180 185 190

Ser Phe Met Trp Met Met Trp Tyr Trp Gly Pro Ser Leu Tyr Ser Ile  
195 200 205

Leu Ser Pro Phe Leu Pro Leu Leu Pro Ile Phe Phe Cys Leu Trp Val  
210 215 220

Tyr Ile  
225



## INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU03/00111

**A. CLASSIFICATION OF SUBJECT MATTER**Int. Cl.<sup>7</sup>: C12N 7/00, 7/01; C12Q 1/70; A61K 39/00

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

SEE BELOW

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  
SEE BELOW

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPIDS, CA, MEDLINE: hepatitis b virus, DNA polymerase, surface antigen, lamivudine, entecavir, baculovirus, mutant, variant

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 01 57244 A1 (MELBOURNE HEALTH ET AL) 9 August 2001 See in particular claims 1, 9, 23 and 24, figure 2 and examples	1-11, 13, 14, 16-29, 32, 35, 36, 38, 39
X	WO 01 94559 A1 (MELBOURNE HEALTH ET AL) 13 December 2001 See in particular claims 24 and 25 and examples	1, 4-9, 17, 30, 31, 32, 35, 36, 38, 39

☒ Further documents are listed in the continuation of Box C ☒ See patent family annex

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier application or patent but published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search  
25 February 2003

Date of mailing of the international search report

05 MAR 2003

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Authorized officer

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 21317 A1 (WESTERN HEALTH CARE NETWORK) 22 May 1998 See in particular example and claims	1, 2, 4, 6, -10, 17, 32, 35, 36, 38, 39
X	Ono SK et al "The polymerase LS28M mutation cooperates with nucleotide binding-site mutations, increasing hepatitis B virus replication and drug resistance" J Clin Invest (2001) 107(4), pages 449-55 See whole document	1-3, 6, 8-12, 17, 22, 32, 35, 36, 38, 39
X	Xiong X et al "In vitro evaluation of hepatitis B virus polymerase mutations associated with famciclovir resistance" Hepatology (2000) 31(1), pages 219-224 See whole document	1, 6, 7, 9, 11, 32, 35, 36, 38, 39
X	Ono-Nita SK et al "YMDD motif in hepatitis b virus DNA polymerase influences on replication and lamivudine resistance: a study by in vitro full-length viral DNA transfection" Hepatology (1999) 29(3), pages 939-45 See whole document	1, 4, 6-9, 17
X	Cane PA et al "Analysis of hepatitis B virus quasiespecies changes during emergence and reversion of lamivudine resistance in liver transplantation" Antiviral Therapy (1999) 4, pages 7-14 See whole document	1-11, 13, 14, 16-21, 32, 35, 36, 38, 39
X	Oon CJ et al "Hepatitis B virus variants with lamivudine-related mutations in the DNA polymerase and the 'a' epitope of the surface antigen are sensitive to ganciclovir" Antiviral Research (1999) 41, pages 113-8 See whole document	1, 6-9, 17
X	Delaney WE and Isom HI "Hepatitis B virus replication in human HepG2 cells mediated by hepatitis B virus recombinant baculovirus" Hepatology (1998) 28(4), pages 1134-46 See whole document	22-25
X	Delaney WE et al "Cross-resistance testing of antihepadnaviral compounds using novel recombinant baculoviruses which encode drug-resistant strains of hepatitis B virus" Antimicrobial Agents and Chemotherapy (2001) 45(6), pages 1705-13 See whole document	22-25, 32, 35, 36, 38, 39

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU03/00111

**Box I** Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos :  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☒ Claims Nos : Claims 32, 35 and 36 in part.  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:  
The claims are not restricted to the technical features of the invention, these being hepatitis B variants, methods of using these variants and products of these methods. The claims have only been searched in so far as they relate to HBV nucleic acid or peptides sequences, antibodies, ribozymes and antisense that are capable of inhibiting the variant HBVs of the invention.
3. ☐ Claims Nos :  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

**Box II** Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. Claims 1-21 and 26-39. These claims recite HBV variants and their uses.
2. Claims 22-25. These claims recite a method of testing for anti-viral agents using recombinant systems that express the HBV genome.

The feature shared by these two sets of claims is HBV variants that may have altered susceptibility to the nucleoside analogues lamivudine and/or entecavir. However this feature is known (see any one of the citations listed).

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims
2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

## INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/AU03/00111

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report			Patent Family Member	
WO	01 57244	EP	1257661	
WO	01 94559	NONE		
WO	98 21317	AU	37628/97	EP 964916

END OF ANNEX



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2260 HV Rijswijk (ZRH)  
☎ (070) 3 40 20 40  
FAX (070) 3 40 30 16

Europäisches  
Patentamt

European  
Patent Office

Office européen  
des brevets

Generaldirektion 1

Directorate General 1

Direction générale 1

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Intellectual Property Department,  
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Industriepark Zwijnaarde 7,  
Box 4  
9052 Gent  
BELGIQUE



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Tel.: +31 (0)70 340 45 00

12-12-2005

05-12-2005

Date  
12.12.05

Reference EP 171	Application No./Patent No. 05101997.4 - 2406
Applicant/Proprietor Innogenetics N.V.	

### COMMUNICATION

The European Patent Office herewith transmits as an enclosure the European search report (under R. 44 or R. 45 EPC) for the above-mentioned European patent application.

If applicable, copies of the documents cited in the European search report are attached.

- ☐ Additional set(s) of copies of the documents cited in the European search report is (are) enclosed as well.

The following specifications given by the applicant have been approved by the Search Division :

- ☒ Abstract ☒ Title
- ☐ The abstract was modified by the Search Division and the definitive text is attached to this communication.

The following figure will be published together with the abstract : none

### Refund of search fee

If applicable under Article 10 Rules relating to fees, a separate communication from the Receiving Section on the refund of the search fee will be sent later.





DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
X	WO 03/087351 A (MELBOURNE HEALTH; AUSTIN & REPATRIATION MEDICAL CENTRE; SOUTHERN HEALT) 23 October 2003 (2003-10-23) * page 8, line 28 * * claim 158 *	1-10	C07K14/02 C12N7/00
A,D	WO 2004/031224 A (GILEAD SCIENCES, INC; DELANEY, WILLIAM, E; XIONG, XIAOFENG) 15 April 2004 (2004-04-15) * the whole document *	1-10	
A,D	BARTHOLOMEW M M ET AL: "Hepatitis-B-virus resistance to lamivudine given for recurrent infection after orthotopic liver transplantation" LANCET THE, LANCET LIMITED. LONDON, GB, vol. 349, no. 9044, 4 January 1997 (1997-01-04), pages 20-22, XP004843545 ISSN: 0140-6736 * the whole document *	1-10	
			TECHNICAL FIELDS SEARCHED (IPC)
A,D	LING R ET AL: "SELECTION OF MUTATIONS IN THE HEPATITIS B VIRUS POLYMERASE DURING THERAPY OF TRANSPLANT RECIPIENTS WITH LAMIVUDINE" HEPATOLOGY, WILLIAMS AND WILKINS, BALTIMORE, MD, US, vol. 24, no. 3, September 1996 (1996-09), pages 711-713, XP002044247 ISSN: 0270-9139 * the whole document *	1-10	C12N C07K
-/-			
The present search report has been drawn up for all claims			
Place of search: Munich		Date of completion of the search: 25 November 2005	Examiner: Herrmann, K
CATEGORY OF CITED DOCUMENTS		T: theory or principle underlying the invention E: earlier patent document, but published on, or after the filing date D: document cited in the application L: document cited for other reasons O: technological background &: member of the same patent family, corresponding document	
X: particularly relevant if taken alone Y: particularly relevant if combined with another document of the same category A: technological background O: non-written disclosure P: intermediate document			



DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
A, D	PERRILLO ROBERT ET AL: "Adefovir dipivoxil added to ongoing lamivudine in chronic hepatitis B with YMDD mutant hepatitis B virus." GASTROENTEROLOGY: JAN 2004, vol. 126, no. 1, January 2004 (2004-01), pages 81-90, XP002355994 ISSN: 0016-5085 * the whole document *	1-10	
A	WO 03/066841 A (MELBOURNE HEALTH; AUSTIN AND REPATRIATION MEDICAL CENTRE; SOUTHERN HEA) 14 August 2003 (2003-08-14) * the whole document *	1-10	
A, D	STUYVER L J ET AL: "NOMENCLATURE FOR ANTIVIRAL-RESISTANT HUMAN HEPATITIS B VIRUS MUTATIONS IN THE POLYMERASE REGION" HEPATOLOGY, WILLIAMS AND WILKINS, BALTIMORE, MD, US, vol. 33, no. 3, March 2001 (2001-03), pages 751-757, XP009029630 ISSN: 0270-9139 * the whole document *	1-10	
			TECHNICAL FIELDS SEARCHED (IPC)
The present search report has been drawn up for all claims			
Place of search: Munich		Date of completion of the search: 25 November 2005	Examiner: Herrmann, K
CATEGORY OF CITED DOCUMENTS		T: theory or principle underlying the invention E: earlier patent document, but published on, or after the filing date D: document cited in the application L: document cited for other reasons &: member of the same patent family, corresponding document	
X: particularly relevant if taken alone Y: particularly relevant if combined with another document of the same category A: technological background O: non-written disclosure P: intermediate document			



### CLAIMS INCURRING FEES

The present European patent application comprised at the time of filing more than ten claims.

- ☐ Only part of the claims have been paid within the prescribed time limit. The present European search report has been drawn up for the first ten claims and for those claims for which claims fees have been paid, namely claim(s):
- ☒ No claims fees have been paid within the prescribed time limit. The present European search report has been drawn up for the first ten claims.

### LACK OF UNITY OF INVENTION

The Search Division considers that the present European patent application does not comply with the requirements of unity of invention and relates to several inventions or groups of inventions, namely:

- ☐ All further search fees have been paid within the fixed time limit. The present European search report has been drawn up for all claims.
- ☐ As all searchable claims could be searched without effort justifying an additional fee, the Search Division did not invite payment of any additional fee.
- ☐ Only part of the further search fees have been paid within the fixed time limit. The present European search report has been drawn up for those parts of the European patent application which relate to the inventions in respect of which search fees have been paid, namely claims:
- ☐ None of the further search fees have been paid within the fixed time limit. The present European search report has been drawn up for those parts of the European patent application which relate to the invention first mentioned in the claims, namely claims:



**ANNEX TO THE EUROPEAN SEARCH REPORT  
ON EUROPEAN PATENT APPLICATION NO.**

EP 05 10 1997

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report. The members are as contained in the European Patent Office EDP file on  
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

25-11-2005

Patent document cited in search report		Publication date		Patent family member(s)	Publication date
WO 03087351	A	23-10-2003	EP	1499712 A1	26-01-2005
WO 2004031224	A	15-04-2004	AU	2003279103 A1	23-04-2004
			CA	2500673 A1	15-04-2004
			EP	1546190 A2	29-06-2005
WO 03066841	A	14-08-2003	CA	2475446 A1	14-08-2003
			CN	1639324 A	13-07-2005
			EP	1476543 A1	17-11-2004
			JP	2005516617 T	09-06-2005



This application is covered by the extended European search report pilot project at present running within the European Patent Office, applied to all European patent applications filed as first filing and searched on or after 01.07.03. Under this project the EPO issues together with the search report an opinion on whether the application and the invention to which it relates meet the requirements of the EPC. This non-binding opinion is issued free of charge as a service. This opinion may be used as the basis for an informed decision as to whether it is desired to pursue the application further or not.

For further details of this pilot project, the applicant's attention is directed to the Official Journal edition 5/2003. If any further immediate questions or comments arise the EPO Customer Services: +31-70-340 4500 or +49-89-2399 2828 can be contacted.

**The attached opinion reveals that the application or the invention to which it relates appear not to meet the requirements of the Convention (see comments on enclosed Form 2906).**

If the applicant wishes to continue with this application the examination fee must be paid. Where appropriate amendments can be filed to address the objections raised in the opinion, thus shortening the overall procedure. If no amendments are filed, the opinion will be re-issued as the first official communication under Article 96(2) and Rule 51(2) EPC.

If the examination fee has already been paid and the right to the communication under Article 96(1) EPC has been waived for this application, the first official communication under Article 96(2) and Rule 51(2) EPC will be issued promptly.



The examination is being carried out on the **following application documents**:

**Description, Pages**

1-46 as originally filed

**Sequence listings, Pages**

1-8 received on 12.05.2005 with letter of 10.05.2005

**Claims, Numbers**

1-27 as originally filed

**Drawings, Sheets**

1/6-6/6 as originally filed

**1 Citations**

The documents mentioned in this communication are numbered as in the European Search Report, i.e. **D1** and **D7** correspond to the first and the last document of the search report, respectively.

**2 Clarity (Art. 84 EPC)**

A polynucleotide may be clearly defined by an amino acid sequence (polynucleotide



encoding the protein according to SEQ ID NO:10). However, this is not the case vice versa. A practically infinite number of (poly)peptides can be deduced from a polynucleotide (6 reading frames, varying lengths). The wording "a(ny) HBV expression product from an isolated HBV polynucleotide..." encompasses any such peptides. Does the Applicant intend to claim all peptides which can be deduced from said polynucleotide? Besides, the claimed peptides must have a defined specific and credible function in order to meet the requirements of Art. 56 EPC.

### 3 Novelty (Art. 54 EPC)

- 3.1 The subject-matter of claims 1-10 does not meet the requirements of Art. 52(1) and 54 EPC.
- 3.2 On p. 8, l. 28 **D1** (WO03087351) discloses mutants at position 181 of the HBV DNA polymerase gene: "Useful mutants are provided below (see also Tables 16 and 17): A181R/N/D/C/Q/E/G/H/I/L/K/M/F/P/S/T/W/Y/V/deletion". Among others, the mutation A181S is disclosed (also cf. claim 158 of **D1**). The subject-matter of claim 1 can thus not be regarded as novel. The subject-matter of claim 2-10 can also not be regarded as meeting the requirements of Art. 54 EPC in view of **D1**.
- 3.3 "A fragment" of a polynucleotide can be as short as one, two or three nucleotides. Thus, even the codons for serine (AGC, AGT, TCA TCC, TCG, TCT) fall under the scope of claim 6 and can thus not be regarded as novel (Art. 54 EPC). Besides, "a fragment" without a defined specific and credible function cannot be regarded as allowable under Art. 56 EPC. The same objections apply to claims 7, 8 and 10.
- 3.4 Furthermore, attention is drawn to the fact that claim 10 is a so-called "product-by-process" claim ("obtainable from"). The EPO considers that "product-by-process" claims are only admissible if the products as such fulfill the requirements for patentability, i.e., inter alia they are novel and inventive and there is no other information available in the application which could enable the Applicant to define the product satisfactorily by reference to its composition, structure or some other testable parameter (cf. T150/82, OJ 7/1984, 309, headnote, point 2).